EFFECTS OF PARASITOSIS (COCCIDIOSIS AND HELMINTHIASIS) AND VIRAL DISEASES (MYXOMATOSIS AND RABBIT HAEMORRHAGIC DISEASE) ON THE PHYSIOLOGICAL CONDITION AND POPULATION DYNAMICS OF THE WILD EUROPEAN RABBIT (*ORYCTOLAGUS CUNICULUS*)

PhD Thesis

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PhD Thesis

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Estación Biológica Doñana
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Memoria presentada por la Licenciada en Biología,

Isabel Pacios Palma

para optar al título de Doctor por la Universidad Pablo de Olavide.

Directores: Sacramento Moreno Garrido y Carlos Rouco Zufiaurre

Tutor: Pedro Jordano Barbudo

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Dra. Sacramento Moreno Garrido, investigadora en la Estación Biológica de Doñana (EBD-CSIC) y el Dr. Carlos Rouco Zufiaurre, investigador postdoctoral en la Universidad de Córdoba

CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral: "Effects of parasitosis (coccidiosis and helminthiasis) and viral diseases (myxomatosis and rabbit haemorrhagic disease) on the physiological condition and population dynamics of the wild European rabbit (*Oryctolagus cuniculus*)", son aptos para ser presentados por la Lda. Isabel Pacios Palma ante el Tribunal que en su día se designe, para aspirar al grado de Doctor por la Universidad Pablo de Olavide.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 13 de Noviembre de 2017.

Firmado

Directores : Dra. Sacramento Moreno Garrido

Dr Carlos Rouco Zufiaurre

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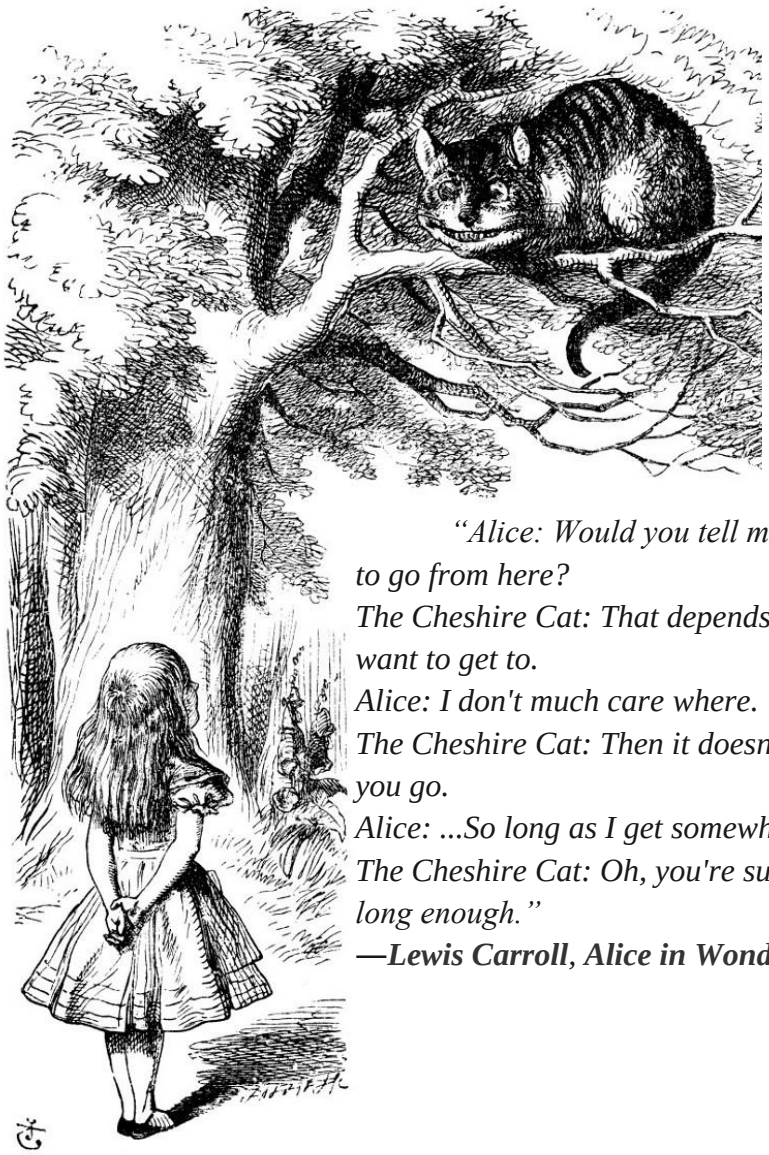
Rabbit's captures and sampling were carried out with all the necessary permits and using ethical consent procedures approved by the Doñana Biological Station Ethical Committee.



"Alice: Would you tell me, please, which way I ought to go from here?
The Cheshire Cat: That depends a good deal on where you want to get to.
Alice: I don't much care where.
The Cheshire Cat: Then it doesn't much matter which way you go.
Alice: ...So long as I get somewhere.
The Cheshire Cat: Oh, you're sure to do that, if only you walk long enough."
—Lewis Carroll, Alice in Wonderland

The Cheshire Cat: Oh, by the way, if you'd really like to know, he went that way.
Alice: Who did?
The Cheshire Cat: The White Rabbit.
Alice: He did?
The Cheshire Cat: He did what?
Alice: Went that way.
The Cheshire Cat: Who did?
Alice: The White Rabbit.
The Cheshire Cat: What rabbit?
Alice: But didn't you just say - I mean - Oh, dear.
The Cheshire Cat: Can you stand on your head?
Alice: Oh!

—Lewis Carroll, Alice in Wonderland

INDEX

The present PhD thesis comprises the following sections: an **abstract** of the thesis, in both languages, English and Spanish; a **general introduction** of the main subject of study with a detailed framework; the general **objectives** of the thesis; a **general materials and methods** section with common methodology used in all chapters ; four **chapters**, each chapter is edited with the format of a scientific article: abstract, introduction, materials and methods, results and discussion, specific for each chapter; a **general discussion** that compiles every relevant result throughout this thesis; and finally the section of **conclusions.**

ABSTRACT

The wild European rabbit (*Oryctolagus cuniculus*) is one of the most important vertebrate species in the Mediterranean ecosystem. It is the staple prey of a wide variety of predators, including some seriously threatened species, such as the Iberian lynx (*Lynx pardinus*) and the Iberian Imperial eagle (*Aquila adalberti*).

Despite wild rabbit populations have been historically numerous and widespread, over the last 60 years they have experienced a sharp decline in the Iberian Peninsula. This decrease was mainly due to the arrival of two viral diseases, myxomatosis and rabbit haemorrhagic disease (RHD). In addition, the effects of these diseases become worsen due to other factors such as habitat loss and fragmentation, and hunting pressure, among others.

Currently these diseases are endemic, causing annual outbreaks with substantial impact on natural rabbit populations. Certainly, emerging and re-emerging infectious diseases play an important role in the population dynamics of wild European rabbit. Undoubtedly, they are one of the main threats for the conservation of this species. Despite of many years of research, there are still gaps in the knowledge of dynamics of viral agents within the European rabbit, their interaction with other parasites and, their impact on life-history traits and biological fitness of individuals. Hence, medium or long-term epidemiological studies and the use of alternative methodologies within wild rabbit populations are of great importance to determine physiological condition of the individuals.

In this sense, the present PhD thesis is aimed at improving our knowledge of eco-epidemiological parameters in wild European rabbit populations facing the two main viral diseases previously mentioned (myxomatosis and RHD). For this purpose, from 2008 to 2010 we conducted seasonal live-trapping sessions in three rabbit

populations under seminatural conditions. The framework of this study was within a project focused on the scientific monitoring and enhancement of wild European rabbit populations in the Sierra de Hornachuelos Natural Park. During this two-year period we collected blood samples to determine serum concentrations of antibodies against myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) as well as to measure biochemical and oxidative stress parameters as condition biomarkers. Rabbit abundance and parasite loads (coccidia and nematodes) were monitored too.

Definitely, this long-term monitoring provides suitable conditions to study the emergence and development of such diseases on wild populations. Furthermore, interactions between pathogens with other biotic factors can be identified, as well as the ultimate effects on the rabbit population dynamics.

RESUMEN

El conejo de monte (*Oryctolagus cuniculus* L.) constituye indiscutiblemente una de las especies de vertebrados más importantes que existe en los ecosistemas mediterráneos. Es la presa principal de un gran número de depredadores, incluyéndose entre éstos especies gravemente amenazadas como el lince ibérico (*Lynx pardinus*) y el águila imperial ibérica (*Aquila adalberti*).

Si bien es una especie ampliamente distribuida y muy abundante, en los últimos 60 años sus poblaciones han experimentado un descenso acusado en la península ibérica. Este declive se debió fundamentalmente a la llegada de dos enfermedades virales, la mixomatosis y la enfermedad hemorrágica vírica del conejo (EHVC). El efecto de las enfermedades se vio potenciado por otros factores tales como la pérdida y fragmentación del hábitat o la presión de depredación entre otros. Actualmente, dichas enfermedades son endémicas y causan brotes epidémicos anuales que tienen un impacto considerable en las poblaciones naturales. Definitivamente, las enfermedades emergentes y reemergentes desempeñan un papel relevante en la dinámica poblacional del conejo de monte y son por tanto una de las principales amenazas para la conservación de esta especie. Sin embargo, a pesar de muchos años de estudio aún existen lagunas en el conocimiento de la dinámica de estos agentes virales en el conejo de monte, su interacción con otros parásitos, y su impacto sobre los ciclos biológicos y el estado físico de los individuos. De ahí la importancia de realizar seguimientos epidemiológicos a medio-largo plazo en las poblaciones silvestres, así como de testar metodologías alternativas para determinar de manera fiable el estado fisiológico de los individuos.

En este sentido, la presente tesis está dirigida a la mejora de nuestro conocimiento de los parámetros eco-epidemiológicos en poblaciones silvestres del

conejo de monte expuestas a las dos principales enfermedades virales anteriormente mencionadas (mixomatosis y EHVC). Para ello, desde 2008 a 2010 se llevaron a cabo capturas periódicas en tres poblaciones de conejo de monte en condiciones seminaturales. Este estudio se llevó a cabo como parte de un proyecto de investigación destinado al seguimiento científico y potenciación de las poblaciones de conejo de monte en el Parque Natural Sierra de Hornachuelos. Durante los dos años de estudio se tomaron muestras sanguíneas para determinar las concentraciones de anticuerpos frente al mixoma virus (MV) y al virus de la enfermedad hemorrágica del conejo (EHVC), además de medir parámetros bioquímicos y de estrés oxidativo como indicadores de condición. Se monitorizó también la abundancia de conejos y las cargas de parásitos (coccidios y nematodos).

Este seguimiento a largo plazo proporciona unas condiciones idóneas para el estudio de la emergencia y desarrollo de enfermedades en poblaciones silvestres. Del mismo modo, también permite identificar posibles interacciones entre patógenos con otros factores bióticos además de determinar las consecuencias últimas sobre la dinámica de población del conejo.

GENERAL INTRODUCTION

The subject of study: the wild European rabbit

The wild European rabbit (*Oryctolagus cuniculus* L.) is a native species to the Iberian Peninsula (IP) (Monnerot et al., 1994). The prolific character of the species, along with human-mediated expansions, has turned the rabbit into a successful colonizer (Thomson and King, 1994). Indeed, the European rabbit is distributed worldwide occupying every continent except for Antartica and in more than 800 islands all over the world (Flux, 1994). First fossil records of *Oryctolagus* genus were found in Southern Spain and date from the Mid-Late Pleistocene around a million years old (Corbet, 1994). During this period two different species existed in the IP: *Oryctolagus laynensis* and *Oryctolagus lacosti*, being the first one the ancestor of the current *O.cuniculus* (López-Martínez, 1989). Late-Pleistocene glaciations split the distribution area of rabbits in two main refugia that were geographically isolated: one located in the southern part of the IP and the other in northern IP and southeast of France (Biju-Duval et al., 1991; Monnerot et al., 1994). Therefore, as a result of these glacial refuge areas, two different rabbit subspecies exist currently: *O.c.cuniculus* and *O.c.algirus.* The former is larger than *O.c. algirus* subspecies and is distributed in the Northeast of IP (Branco et al., 2000, 2002; Esteves et al., 2006, Alves and Hackländer, 2008; Carneiro et al., 2009; Ferreira et al., 2015), whereas *O.c. algirus* is mainly restricted to the south and west of IP (Atkinson et al., 2007) (Figure 1). *O.c.cuniculus* was expanded all over the world and originated the domestic breeds of rabbits that we know to date.

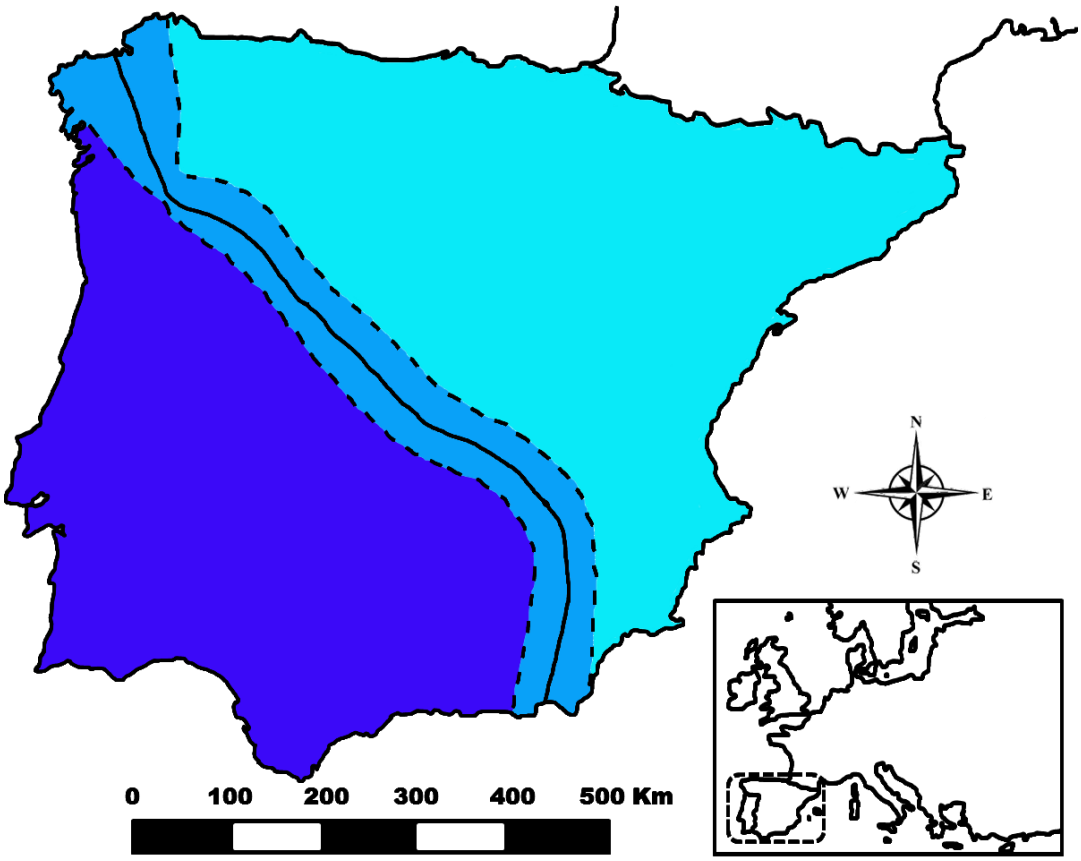


Figure 1.Geographical distribution of the wild European rabbit subspecies in the Iberian Peninsula: Oryctolagus cuniculus cuniculus (in cyan), Oryctolagus cuniculus algirus (in bright blue) and hybrid or coexistence zone (in light blue). (Source modified from Carneiro et al., 2009).

Biology of the wild European rabbit

The wild European rabbit is a small-sized mammal (average weight between 800 and 1300 g) that belongs to the Family Leporidae within the Order Lagomorpha. Despite of the notable adaptability displayed, European rabbit populations are difficult to find above 1500 m high (Villafuerte, 2002). Remarkably, rabbits' social structure is extremely complex with a strong hierarchy among them that determine ultimately their reproductive success. They constitute polygamous groups which consist of a dominant male and several reproductive females together with juveniles and other subordinated

males (Lockley, 1961; Cowan and Garson, 1985). Social grouping is basically determined by habitat quality and the availability of natural resources.

Other factors such as temperature, gradient, altitude, rainfall and type of soil play an important role in the abundance and distribution of populations at a regional scale (Trout et al., 2000; Calvete et al., 2004a; Saldaña et al., 2007). In the IP, European rabbit prefers habitats with sparse Mediterranean scrublands interspersed with good pastures and/or crops that provide them with food and sheltered areas against predators (Rogers and Myers, 1979; Wheeler et al., 1981; Lombardi et al., 2003; Carvalho and Gomes, 2004; Delibes-Mateos et al., 2009).

Also, warren-digging is an especially critical strategy for the survival of the European rabbit. To build such structures, they prefer dry, well-drained, soft and light-compacted soils. In this sense, warrens represent an important element for protection from predators, climatic extremes and, they play a crucial role in breeding (Hall and Myers, 1978; Parer and Libke, 1985).

Rabbits are characterized generally by a crepuscular circadian rhythm although it has been observed they can modify patterns of activity depending on the season and/or local conditions (Rogers et al., 1994). They spend most of the time foraging and feeding on a wide variety of plants including predominantly green grasses, legumes and small-sized cereals. Preferably, they select soft plants rich in nitrogen and low in fiber (Thompson, 1994; Myers and Bults, 1977) but they can adjust their diet according to the location or climate conditions to suit phenology and development of vegetation (Chapuis, 1990). As the food they consume has a high content in fiber, rabbits present an adaptation in their digestive system to optimize the intake of low-quality food, i.e: caecotrophy. Caecotrophy is a physiological mechanism present in leporids that consist

in the consumption of faecal pellets (so called caecotrophes) which are rich in vitamins and proteins (Hirakawa, 2001).

The main reason for the great ecological success of the European rabbit is the elevated reproductive capacity. Moreover, rabbit is a well-adapted animal that shows a high sensitivity to climatic conditions and to the availability of food at local scales. This characteristic let it adjust the reproductive cycle, taking advantage of the most favourable periods for breeding (Wheeler and King, 1985; Gonçalves et al., 2002). This way the beginning, the duration and the intensity of the breeding season are subjected to significant variability every year. Generally, in the IP rabbit's breeding season goes from January to May and the gestation period is around 30 days. Female rabbits show early sexual maturity, being able to reproduce since 4 months and producing big-sized litters (around 3-6 kittens/female). Additionally, females can be sexually receptive during all year, so they are likely to produce several litters in only one reproductive season (Rogers et al., 1994).

The wild European rabbit: a keystone species in the Mediterranean ecosystem

The wild European rabbit plays an important role as a key species in Mediterranean ecosystems (Delibes-Mateos et al., 2007, 2008). Traditionally considered the most widespread mammal along the IP with high-density populations, it has become the main prey of a wide array of predators. Most of them are raptors and mammalian carnivores of high conservation value (Delibes and Hiraldo, 1981) that includes two highly endangered vertebrates: the Iberian lynx (*Lynx pardinus*) and the Iberian imperial eagle (*Aquila adalberti*) (Delibes and Hiraldo, 1981, Delibes-Mateos et al., 2007) (Figure 2). These Iberian species prey almost exclusively upon rabbits, so the European

rabbit plays a pivotal role for the conservation of both predators (Ferrer and Negro, 2004).

Moreover, the European rabbit is probably the most important small-game species in the IP (Delibes-Mateos, 2006) (Figure 2), as it has been proved with archeological sites (more than 65000 years old) and also historical hunting data (Hockett and Bicho, 2000; Hockett and Haws, 2002; Stiner and Munro, 2002; Biadi and Le-Gall, 1993). Classically considered a really prolific species, it has been largely appreciated as a source of food and fur.

Nevertheless, in the recent decades European rabbit's populations have drastically decreased within their natural areas of distribution (Blanco and Villafuerte, 1993; Villafuerte et al., 1998) as a result of unsustainable hunting (Piorno, 2006), predation pressure (Moreno et al., 2007), habitat loss and fragmentation (Calvete et al., 2004a) but principally due to the strong and devastating impact of diseases (Moreno et al., 2007, Williams et al., 2007).

Likewise, the wild European rabbit is an important ecosystem modeler because of its digging activity (warren building and rabbit scrapes), the deposition of faecal pellets (latrines) and other effects such as grazing and seed dispersal derived from the herbivorous activity. Therefore, it is considered an important ecosystem engineer of Mediterranean ecosystems in the IP (Gálvez-Bravo, 2008; Gálvez et al., 2008; Gálvez-Bravo et al., 2009). In this regard, several authors have showed evidences of rabbits' influence on the abundance and diversity of vegetal communities (Farrow, 1917; Fenton, 1940; Gillham, 1955; Watt, 1981; Crawley, 1990; Crawley and Weiner, 1991; Gómez-Sal et al., 1999; Eldridge and Simpson, 2002; Dendy et al., 2004) (Figure 2).

Aforementioned, the European rabbit is characterized by forming high-density populations in favourable conditions (Palomares, 2001). In addition, rabbits are highly

selective animals concerning food habits, so the vegetal species they prefer are likely to be scarce when they are found in high densities. Contrary, those species less preferred would be very abundant (García-Fuentes et al., 2006). Continuous grazing has been proved to reduce considerably the average height of plants and also favours the existence of open areas of scrubland (pastures generally) with high availability of food and refuge. This is the type of habitat that rabbits generally prefer (Moreno and Villafuerte, 1995). In the IP the generation of ecotone areas by the European rabbit is believed to be beneficial for small mammals and also several predators (Delibes-Mateos et al., 2008).

As others herbivore species, the European rabbit is an important seed consumer and thus it plays an essential role as a dispersal agent. In the Mediterranean region, rabbits are able to disperse seeds from a wide variety of plants such as herbs, bush (e.g. *Retama monosperma*) and even tree species (e.g. *Olea europaea*) (Soriguer, 1986; Muñoz-Reinoso, 1993; Rogers et al., 1994; Malo and Suarez, 1995; Malo et al., 1995; Cerván Carmona and Pardo Navarro, 1997; Malo et al., 2000; Dellafiore et al., 2006). Even though rabbits' faecal pellets have a small proportion of seeds (about 2, 5%), the high daily deposition rate (around 350 faecal pellets per day, Delibes-Mateos et al., 2009a) results in a large number of seeds dispersed during a year (Wood, 1988; Dellafiore et al., 2006). It has been observed that seeds' germination capacity remains intact despite the pass through the rabbit's gastrointestinal tract and what is more, germination potential may be increased in comparison with other frugivorous vertebrates (Cosyns et al., 2005). The European rabbit is characterized by depositing a large number of pellets (Gibb, 1990) that have high concentrations of nitrogen and phosphorus; so depositions have an important influence on physical and chemical properties of the soil (e.g. significant increase of nutrients and organic material in soil,

alterations in pH and the existence of a high diversity plant community with increased total biomass) (Willot et al., 2000; Petterson, 2001).

However, little attention has been paid to the potential role in promoting plant biodiversity through heterogeneous distribution of nutrients and their contribution to soil fertility. In this sense, latrines may constitute “fertile islands” that would facilitate the growth of plants (Garner and Steinberger, 1989).

High warren densities have been reported in areas with large rabbit populations (up to 10 warrens/ha), being a significant element in the Mediterranean landscape (Blanco and Villafuerte, 1993; Gea-Izquierdo et al., 2005). Frequently some species of amphibians, reptiles, mammals, and birds occupy rabbit warrens too (Blas-Aritio, 1970; Blázquez and Villafuerte, 1990; Palomares and Delibes, 1993; Revilla et al., 2001 Gálvez-Bravo, 2008) (Figure 2).

Paradoxically, despite the European rabbit is classified globally as a ‘Near Threatened’ species and as a ‘Vulnerable’ species in Portugal and Spain respectively, by the Red List of Vertebrates according to IUCN criteria (Cabral et al., 2005; Villafuerte and Delibes-Mateos, 2007) , it is as well considered an agricultural pest (Barrio, 2010).

In the recent decades, European rabbit populations have suffered from a dramatic decline in their natural range in the IP. Even so, some populations have experienced a slow recovery (Delibes-Mateos et al., 2009b, 2011) especially in homogeneous patches within semiarid Mediterranean agro-ecosystems where croplands supply food and the remnants of natural vegetation and the edges of fields provide shelter and breeding sites (Calvete et al., 2004a). In such scenarios they are reported to produce local explosions that can destroy native vegetation, damage crops, compete with livestock and native wild species and cause definitely severe ecological impacts (Lees and Bell, 2008; Delibes-Mateos et al., 2017). Therefore, this point possesses a

conflict between hunters, farmers and conservationists in agro-ecosystems because rabbits threaten seriously native ecosystems and thence efficient management strategies need to be discussed. (Delibes- Mateos et al., 2014).

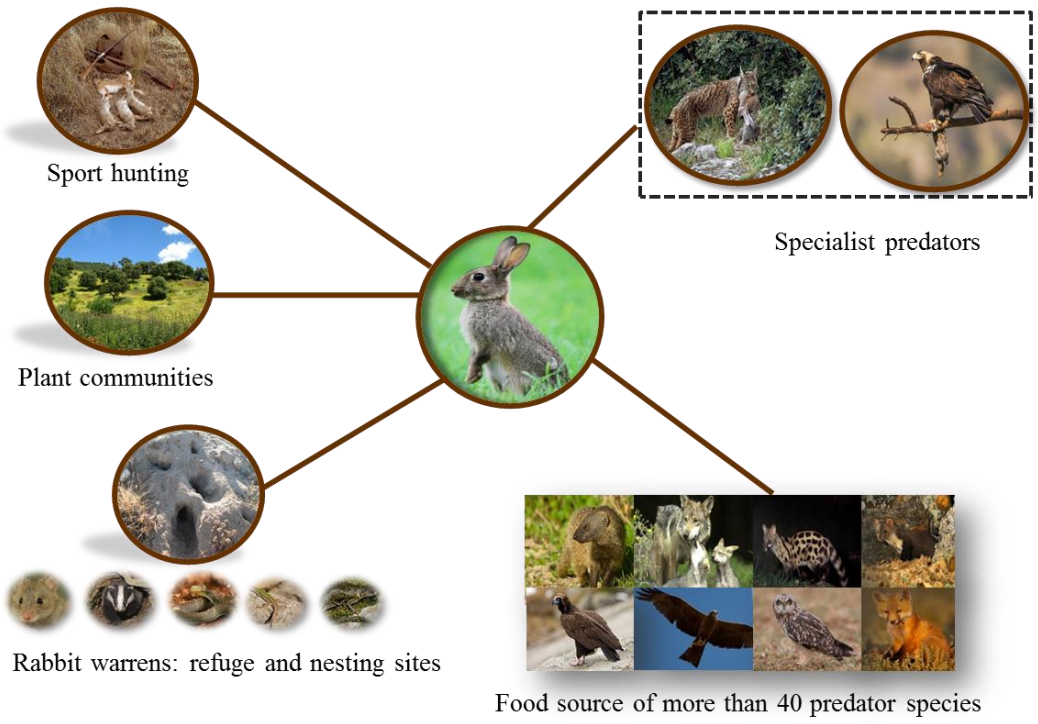


Figure 2. The wild European rabbit: a keystone species in the Mediterranean ecosystem.

Decline of the wild European rabbit populations

As we mentioned before, despite European rabbits have been historically numerous and widespread in the IP their populations have undergone a significant decrease over the last 60 years (Muñoz, 1960; Thompson and King, 1989; Moreno et al., 2007; Delibes-Mateos et al., 2009b). Nevertheless, the effects of this decline were uneven with local extirpations being reported in some areas with still high rabbits' density while in many other rabbit populations they occurred at very low density. The main causes were habitat loss and fragmentation (e.g. due to agriculture, forestry,

development and fires; Ward, 2005), excessive predation pressure (Delibes-Mateos et al., 2007), human-induced mortality (e.g. excessive hunting and rabbit control; Ward, 2005) and mostly the high impact of rabbit diseases (myxomatosis and rabbit haemorrhagic disease; Calvete, 2006).

Additionally, other pathogens like nematodes and coccidia may affect wild populations as well. Most studies have focused on the epidemiology and pathogenesis of these diseases separately; however the potential role of coinfection, not necessarily with both viral diseases but its interaction with other parasites, has been so far neglected.

Viral diseases: myxomatosis

Myxomatosis was first described between the end of the 19th century and the beginning of 20th century in Montevideo (Uruguay), where a lethal infection of imported domestic (laboratory) European rabbits occurred (Sanarelli, 1898; Kessel et al., 1931). After its discovery, the introduction of myxoma virus (MV) was proposed as a biological control mechanism into European rabbit populations that meant serious pests. Finally, it was legally introduced in Australia (Ratcliffe et al., 1952) with a significant success. Then, other countries with rabbit pests such as France, United Kingdom or Argentina decided to introduce the virus illegally in an attempt to manage the problem. Soon after, it spread rapidly throughout the European continent (Fenner and Ross, 1994) and reached the IP in 1953.

First outbreaks caused mortality rates close to 100% (Armour and Thompson, 1955; Lloyd, 1970), decimating populations drastically. During the following years, the mortality remained high but lower than during the initial outbreaks (Fenner and Ratcliffe, 1965) mainly due to the selection of less virulent virus strains but also

because rabbits developed genetic resistance against MV (Sobey, 1969; Best and Kerr, 2000).

Despite most populations recovered from the initial decline, myxomatosis currently has become an enzootic disease in the wild (Fenner and Ross, 1994; Villafuerte et al., 2017) with significant impact on wild European rabbit populations. Myxomatosis is a viral disease caused by a *Leporipoxvirus* belonging to family *Poxviridae* (Fenner and White, 1976) (Figure 3a). MV was originally a natural pathogen of the South American tapeti (*Sylvilagus brasilensis*) and of the North American brush rabbit (*Sylvilagus bruneii*); in its natural host it induces a benign infection characterized by a cutaneous fibroma restricted to the site of inoculation. Nonetheless, it causes a lethal and systemic disease (myxomatosis) in domestic and wild European rabbits (*Oryctolagus cuniculus*) characterized by nodular lesions on face, ears and anogenital regions as well as blepharoconjunctivitis (Fenner and Ratcliffe, 1965; Flohe et al., 1997; Moss, 2001) (Figure 3b). Myxomatosis lethality is believed to be a result from the progressive impairment of the host immune response, since MV replicates inside lymphocytes and thence promote uncontrolled secondary bacterial infections (Heard et al., 1990) or any other parasites such as helminths (Mykytowycz, 1959; Boag, 1988). MV transmission occurs mainly by arthropod vectors such as fleas and/or mosquitoes that bit the host (Fenner and Ratcliffe, 1965). Indeed, rabbit warrens are a key element in this process since they are used for several flea species as refuge areas where they appear in high numbers and act as potential vectors (Osácar-Jiménez et al., 2001).Also direct transmission by contact between infected and non-infected rabbits has been described but is less frequent (Aragao, 1927; Mykytowycz, 1958; Joubert et al., 1972).

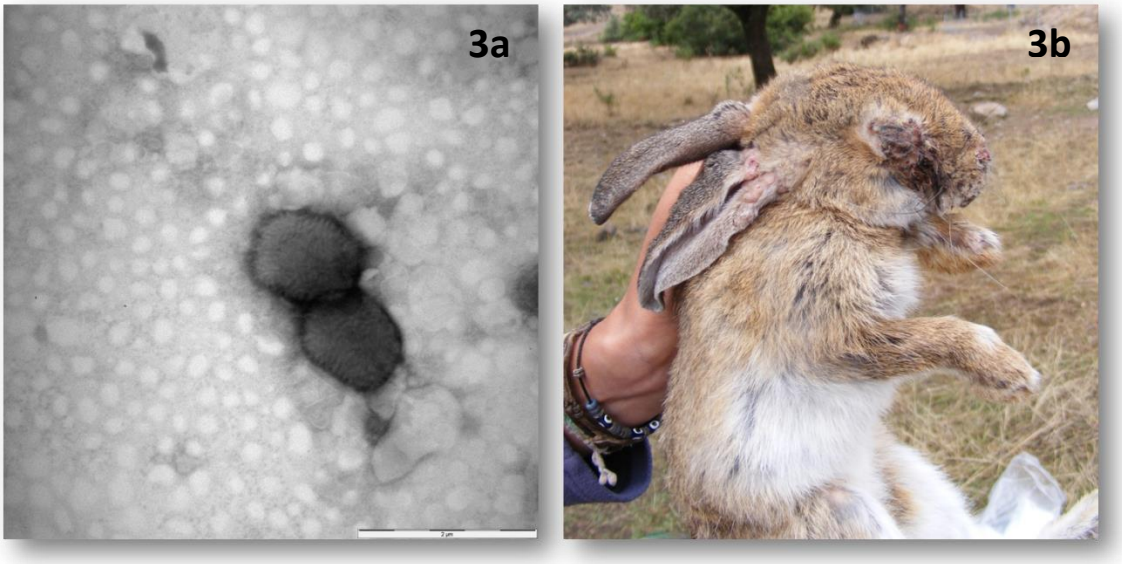


Figure 3. (a)Myxoma virus (TEM images with FEI Tecnai G2 Spirit BioTWIN transmission, stained with 2% NaPT solution). Source provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (I.Z.S.L.E.R.); (b) Rabbit with myxomatosis in Sierra de Hornachuelos Natural Park.

Viral diseases: Rabbit haemorrhagic disease

Rabbit haemorrhagic disease (RHD) was first described in 1984 in a group of commercially bred of Angora rabbits imported from Germany into the Jiangsu Province of the People's Republic of China (Liu et al., 1984). It appeared as an extremely lethal and highly contagious disease that spread rapidly in many European countries. In early summer 1988, RHD was reported in the IP for the first time where it caused high mortality rates close to 80% during the initial outbreaks of the virus (Argüello et al., 1988; Villafuerte et al., 1994). Over the subsequent decades, rabbit population densities suffered a substantial reduction that reached a point close to extinction in some areas (Villafuerte et al., 1995; Calvete, 2006).

However, RHD is currently an endemic disease in the IP and so originates considerable mortalities (over 20%) during summer and winter; seasons when RHD outbreaks occur normally (Calvete et al., 2002; García-Bocanegra et al., 2011). The

disease is constrained mainly to adult individuals (about two months of age) since juvenile rabbits result generally unaffected (Prieto et al., 2000; Ferreira et al., 2008). The aetiological agent of RHD, rabbit haemorrhagic disease virus (RHDV), has been identified as a member of the genus *Lagovirus* within the family *Caliciviridae* (Ohlinger et al., 1990; Parra and Prieto, 1990; Rodak et al., 1990; Meyers et al., 1991; Moussa et al., 1992) (Figure 4a) and affects domestic, farmed and wild European rabbits. This genus also includes other non-pathogenic calicivirus which causes asymptomatic seroconversion in rabbits and has been hypothesised to be a potential ancestor of RHDV (Capucci et al., 1996).

The general pathology of RHDV is typically characterized as an acute hepatitis with hemorrhages and congestions in the liver and also in other organs such as lungs, kidneys and heart resulting from a massive disseminated intravascular coagulation that cause animal's fulminant death. Externally, a variety of symptoms such as anorexia, apathy and congestion of the palpebral conjunctiva, opisthotonos, excitement, paralyses or ataxia may be observed. Some respiratory signs (i.e., tracheitis, dyspnea and cyanosis) and nasal, ocular haemorrhages and, epistaxis may also occur (Mitro and Krauss, 1993; Abrantes et al., 2012) (Figure 4b). After being infected, animals normally experience an incubation period between 16-48h. Then death occurs between 2-3 days post-infection but sometimes can occur several days later. RHDV transmission is mainly through direct contact with an infected animal, since infected rabbits may shed viral particles in their secretions and excretions such as faeces (Ohlinger et al., 1993; Abrantes et al., 2012). Routes for the virus entrance are usually oral, nasal or conjunctival. Additionally, RHDV can be transmitted passively over short distances by blood-feeding insects (i.e: mosquitoes, fleas and, flies) and also it may be spread to new areas through people movement, fomites-contaminated food, bedding, water, clothing,

cages and equipment (Xu and Chen, 1989; Chasey, 1994; Asgari et al., 1998; Frolich et al., 1998; Cooke, 2002). In the field, particularly carcasses of RHDV-infected rabbits are a major source for viral transmission since the virus may be highly resistant and stable even when exposed to harsh environmental conditions. Indeed carcasses of RHDV-infected rabbits have been reported to contain viable viral particles for up to three months (McColl et al., 2002; Henning et al., 2005).

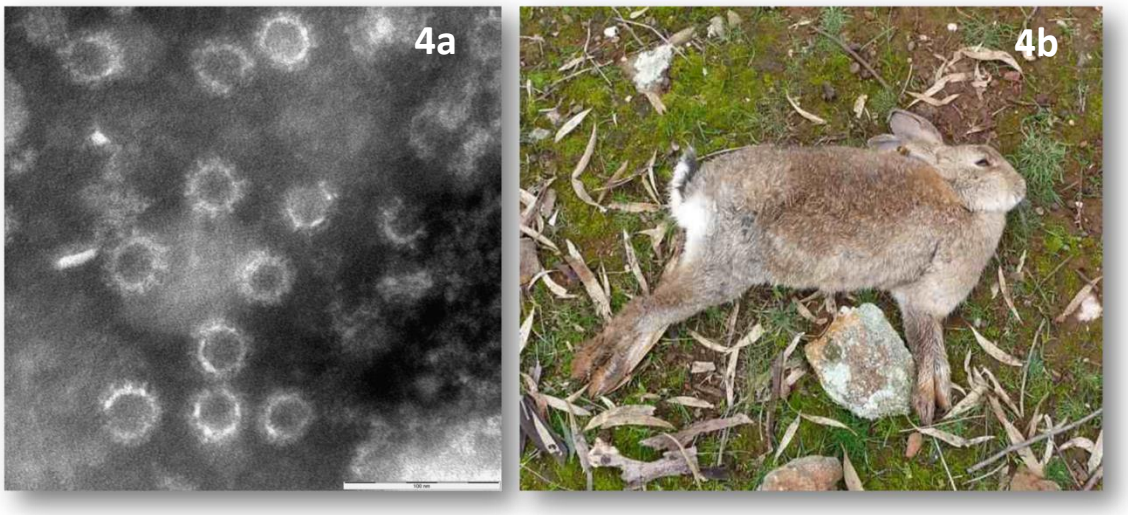


Figure 4. (a) Rabbit haemorrhagic disease virus (TEM images with FEI Tecnai G2 Spirit BioTWIN transmission, stained with 2% NaPT solution). Source provided by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (I.Z.S.L.E.R.) (b)Rabbit found dead of rabbit haemorrhagic disease in Sierra de Hornachuelos Natural Park.

Rabbit haemorrhagic disease: emergence of a new strain

In 2011, a new variant of RHDV that emerged in France in 2010 (Le Gall-Reculé et al., 2011), was detected in Spain for the first time and rapidly spread to several European countries (Dalton et al., 2012; Abrantes et al., 2013; Le Gall-Reculé et al., 2013; Westcott et al., 2014; Baily et al., 2014). This new lagovirus was originally designated as "RHDV2" (Le Gall-Reculé et al., 2013) or "RHDVb" (Dalton et al., 2012), although a recently proposed new nomenclature based on phylogenetic relationships designates it as *Lagovirus europaeus*/GI.2, henceforth GI.2 (Le Pendu et al., 2017). GI.2 shows a unique antigenic profile and atypical features, causing high mortalities in adults but also in kittens (less than two months old). Even vaccinated rabbits against the classical variant, although less susceptible, did not show an efficient complete protection against this new strain.

Despite of this fact, GI.2 is characterized as being less virulent for adult rabbits (up to 75% mortality) in comparison with classical variants of RHDV (above 80% mortality). Remarkably, mortality among kittens younger than 30 days is even higher with GI.2, whereas was basically none with the classical variant. In the present study we did not refer to GI.2 because the data collected came from free-living European rabbit populations monitored from 2008 to 2010 and there was no evidence of the new GI.2 variant over those two years.

Parasitosis: coccidiosis and helminthiasis

Parasitic diseases such as coccidiosis and helminthiasis, together with viral diseases (i.e: MV and RHDV), are considered to cause an important impact on European rabbit populations. Actually, potential interactions between coccidia, nematodes and myxomatosis result in a substantial reduction of rabbits' fecundity and

survival (Boag et al., 2013). Particularly, Cattadori et al. (2007) even stated that the MV interferes with the immune response and this would lead to an increase in helminth and coccidian loads. Host age, sex and reproductive status are some factors likely to affect counts of parasites. The coccidian infections are most frequently caused by *Eimeria* species. Around eleven *Eimeria* species have been reported to parasitize rabbits (Hobbs and Twigg, 1998; Pakandl, 2009) and the majority of them are found in the intestines (Boag et al., 2013). Generally, coccidian infections have been reported to cause severe body weight losses and consequently they can reduce the overall rabbit's physical condition, in fact even more than MV infections (Lello et al., 2005). Remarkably, mortality rates due to coccidian infections are recorded to exceed sometimes mortality due to RHDV (Marchandeau et al., 1999). Coccidiosis occurs commonly in juvenile rabbits that are unlikely to develop immunity to coccidian infections (Mykytowycz, 1962; Bull 1958; Cowan 1983; Oppelt et al., 2010). Also seasonality has been recorded as a central point for coccidian infections because coccidian oocysts need warm temperatures and relatively moist environment for sporulation and survival (Hobbs et al., 1999).

Concerning helminths, we only accounted for nematode species. Particularly, species of the genus *Trichostrongylus*, *Graphidium* and *Nematodiroides* are the most common species that parasitize rabbits. As previously mentioned for coccidia, they also appear in the digestive tract (Foronda et al., 2003). It has been demonstrated also that nematode infections can affect adversely growth rate, body weight and fecundity of rabbits (Bull, 1964; Dunsmore, 1980a, b, c). Thus, nematodes prevalences are mostly associated to humidity and temperature conditions, so lower nematode intensities are expected in areas with extreme climatic conditions (Blasco et al., 1996). Some studies evidenced an effect of sex on the helminth loads with female rabbits presenting higher

prevalences, whereas other authors did not find significant differences between sexes (Boag, 1985; Butler, 1994). It is believed that differences in the levels of parasitation between female and male rabbits are mostly related to seasonality (Bull, 1959; Dunsmore, 1966; Dunsmore and Dudzinski, 1968). Results from Molina et al. (1999) indicated that nematodes in European rabbits are likely to be influenced by host age, occurring more frequently in adults. However, this seems to be controversial since other studies reported the contrary.

Overall, the interaction among several parasites in European rabbits is crucial to host fitness and to the epidemiology of myxomatosis and rabbit haemorrhagic disease. These diseases have caused significant reductions in rabbit populations on the Iberian Peninsula. And despite what mentioned above, most studies have focused on the epidemiology and pathogenesis of these viruses individually, and little is known about interactions between these parasites and the two main viral diseases that affect rabbit.

OBJECTIVES

The present PhD thesis is aimed at studying eco-epidemiological parameters from three wild populations of the European rabbit (*Oryctolagus cuniculus*) kept at seminatural conditions that were naturally exposed to the main viral diseases that affect rabbits: myxomatosis and rabbit haemorrhagic disease RHD). One important point is the effect that these viruses have on rabbit survival, thence we explored if rabbits infected naturally with myxoma virus (MV) and rabbit haemorragic disease virus, (RHDV) that developed anti-MV and anti-RHDV antibodies, increased their survival rates. To address such question we (i) estimate monthly survival rates dependent on serostatus, age (juveniles vs. adults) and sex, (ii) assess monthly seroconversion rates of both diseases according to hosts' age and sex, (iii) quantify the dynamics of seroprevalence to each virus over time, expressed as the seropositive probability and, (iv) examine relationships between population size and seroprevalence (**chapter 1**). Also the interaction of these viruses with other parasites (i.e: coccidia and nematodes) has been described and may affect disease outbreaks and ultimately rabbit population dynamics. Thus we (i) test the influence of coccidia and nematodes on the ability to generate an immunological response against MV and RHDV (ii) analyse the effect of coinfection between MV and RHDV infections and parasite infections and, (iii) identify interactions that may affect physiological condition and get more susceptibility to secondary infections (**chapter 2**). In this sense, long-term monitoring is needed to detect new disease outbreaks and to assess the impact they have on the health status of wild rabbit populations. Recently, there has been a surge in the use of new biomarkers (i.e: blood biochemistry and oxidative stress (OS) makers) as reliable tools to survey physiological alterations that may occur in diseased individuals. In this line we (i) assayed some biochemical parameters and OS biomarkers as proxies of rabbit physiological condition (ii) test if rabbits infected naturally with MV and RHDV that

developed anti-MV and anti-RHDV antibodies showed any alteration in serum biochemistry or an increase OS status and, (iii) determine if rabbits seropositive for MV and/or RHDV presented signs of oxidative damage once they were infected (**chapters 3 and 4,** respectively).

Overall, this study will provide a deeper insight into our knowledge and understanding of a complex epidemiological system.

In summary, we addressed the following goals:

1) Test the effect myxoma virus and rabbit haemorrhagic disease virus seroprevalence on the survival of wild rabbit populations.

2) Study the effects of coccidian and nematode infections on myxoma virus and rabbit haemorrhagic disease virus seroprevalence.

3) Determine the relationship between serum biochemistry and myxoma virus and rabbit haemorrhagic disease virus seroprevalence.

4) Examine whether wild rabbits naturally infected with myxoma virus and rabbit haemorrhagic disease virus had high oxidative stress.

GENERAL MATERIALS AND METHODS

Ethics statement

All animal experimentation was carried out in accordance with Spanish and European regulations (Law 32/2007, R.D. 1201/2005, and Council Directive 2010/63/EU, R.D. 53/2013, ECC/566/2015).

Study area

The study was conducted in Sierra de Hornachuelos Natural Park (100–700 m a.s.l.), which is located in a mountainous area in the southwestern of the Iberian Peninsula (37°49′ N, 5°15′ W) (Figure 5). The climate is Mediterranean, characterised by hot, dry summers and mild, wet winters (Pinilla et al., 1995) (Figure 6)

As mentioned before, this study was framed in a project focused on scientific monitoring and enhancement of European rabbit populations in the Sierra de Hornachuelos Natural Park, which main aim was to increase local rabbit abundance as a prey in order to favour rabbit-specialist endangered predator species through a rabbit restocking program. Rabbits for restocking were originally captured in a natural area 50 km far which ensure to a great extent genetic similarity of donor populations (Branco, 1995). After that, rabbits were transported and released in enclosures over 3-4 ha of extension. Enclosures were surrounded by 2.5-m-high chain-link fence to prevent rabbit emigration and to exclude terrestrial predators (Rouco et al., 2008). Within every enclosure, 30 artificial warrens regularly distributed were built (by using pallets covered by earth and branches) to provide animals with shelter and nesting sites.

Additionally, water and commercial rabbit food were supplied *ad libitum* throughout the study period and grasses were sown to increase the availability of fresh food. Essentially, the major goal of this measure was to create high rabbit density areas, where animals would maximize their productivity and, ultimately become the 'local' donor populations for neighbouring areas for future translocations.

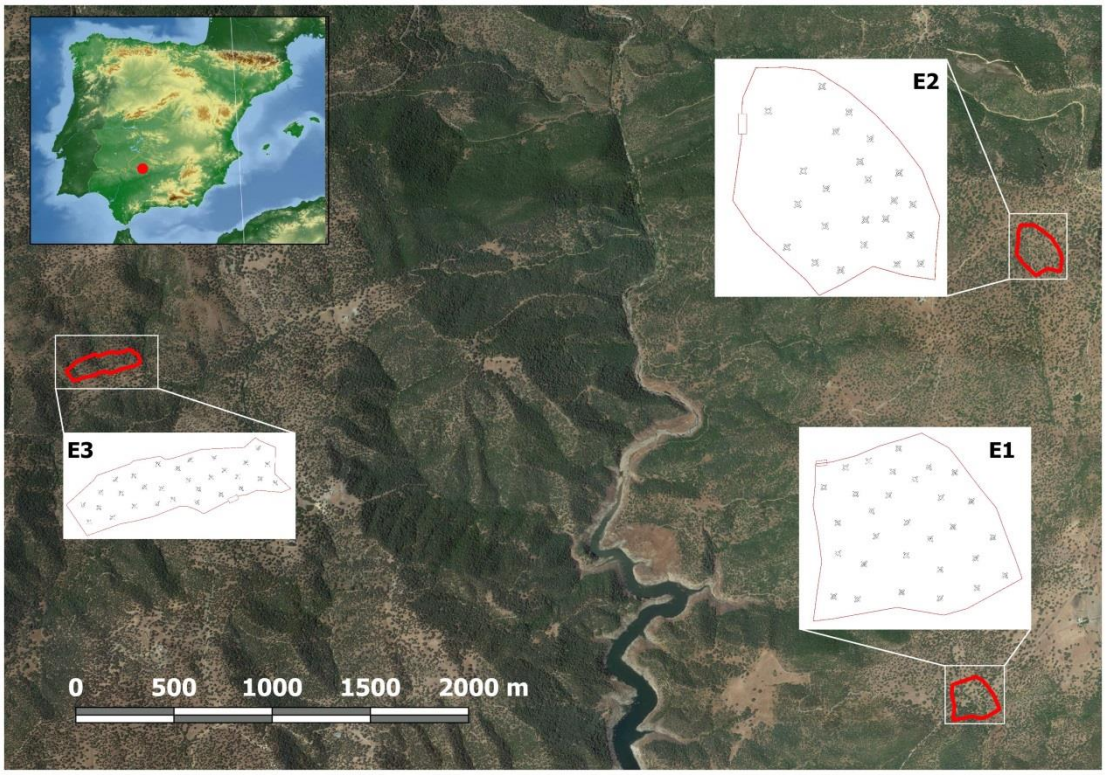


Figure5. Map showing the location of the study area within the Iberian Peninsula and the location of the enclosures studied within the Sierra de Hornachuelos Natural Park. Red polygons represent the rabbit enclosures (E1, E2, and E3). Map of location of artificial warrens within each enclosure is shown.

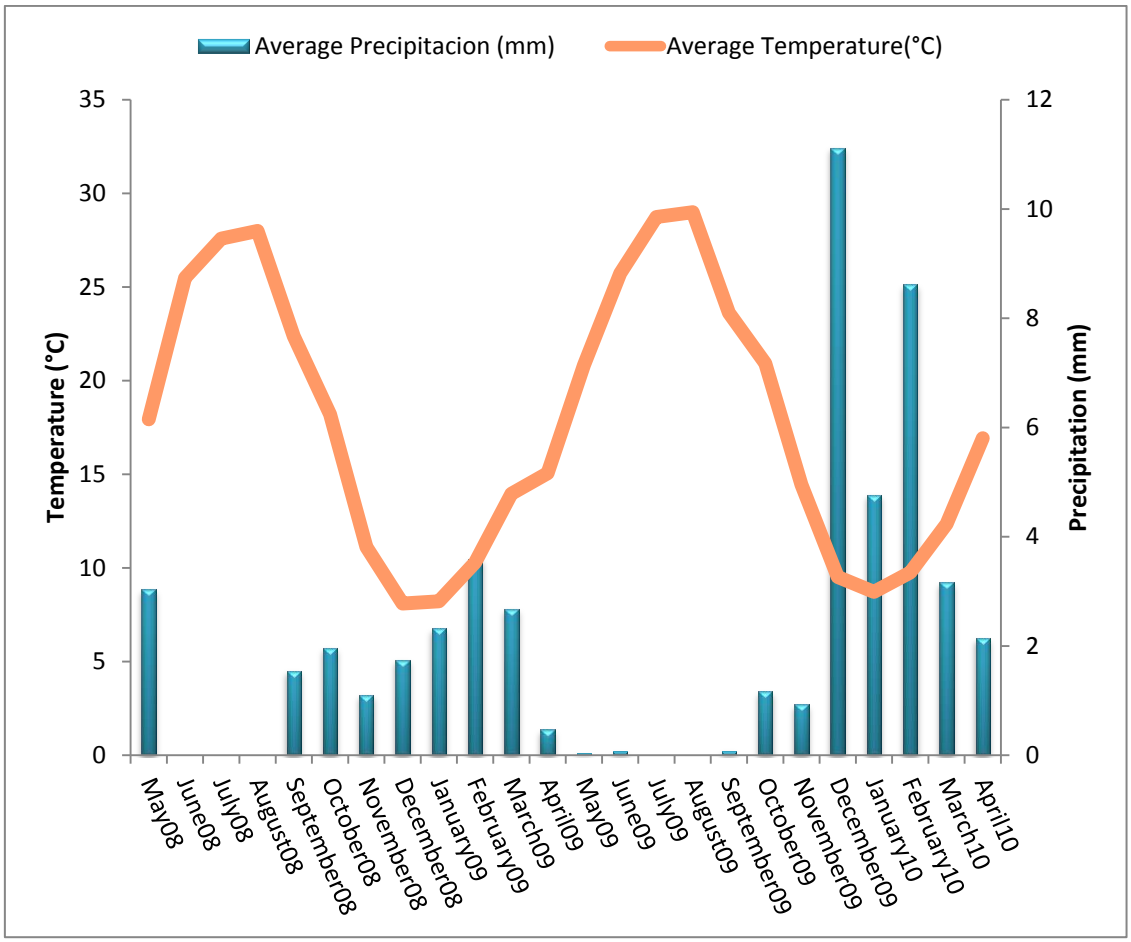


Figure 6. Monthly average precipitation (mm) and average temperature (º C) in the Sierra de Hornachuelos Natural Park (Source: IFAPA, data collected in Hornachuelos weather station) throughout the period of study.

Experimental design

Sampling of rabbit populations

The study was conducted from May 2008 to April 2010 and involved seasonal live-trapping sessions within three wild rabbit restocking enclosures (E1, E2 and E3, Figure 5). Geographically, the distance from one enclosure to another was around 3-4 km. Within them artificial warrens were built with an effective capture device that consisted of a wire net fence with 4 metal cage-traps attached to holes in the fence that acted as doors (Rouco et al., 2011) (Figure 7).

Capture comprised activation of the capture devices at midday (when rabbits show less activity and spend most time underground; Villafuerte et al., 1993). The following morning, rabbits that got trapped inside the cages were counted and handled at the capture site. Remarkably, this trapping system permitted capture of around 50-60% of rabbits within each warren on any one night (Rouco et al., 2011). Every rabbit captured was marked with individually numbered ear tags (National Band & Tag Co. size 3, Kentucky, USA) and its sex and weight to the nearest 25 g were recorded. Additionally biometrical parameters, such as tarsus and ear length, were measured with a caliper to 0.1 mm. Body mass (weight) was used to determine rabbit's age. So, females and males weighing 750 g and 850 g, respectively, were considered adults (Villafuerte et al., 1994; Alves and Moreno, 1996). Furthermore, from the total number of animals captured we selected from 2-8 rabbits per warren and transported them to a field laboratory within each enclosure. Blood samples (1-2.5 mL) were taken from the auricular marginal vein that we kept cold (4 ºC approximately, avoiding direct contact with ice) till centrifugation in Eppendorf tubes. Serum obtained was stored at -80ºC (Evans, 2008; Maceda-Veiga et al., 2015) and analysed afterwards to determine antibody concentrations and other physiological parameters of interest (i.e. biochemistry and oxidative stress parameters) in the Physiological Ecology Laboratory of the Doñana Biological Station (Seville, Spain). In further chapters we will describe these analyses in more detail. Once blood samples were extracted, rabbits were immediately released into the warren of capture.

Figure 7. Trapping device system used in the capture of rabbits compound with a 1) metal cage-trap (1) with a wooden box to provide shelter (2) and two door-tunnel-doors (3), one of them attached to the warren fence.

Serology analysis

To determine concentrations of anti-MV and anti-RHDV antibodies we used commercial enzyme-linked immunosorbent assay (ELISA) kits; the diagnostic techniques recommended by the World Organization for Animal Health (OIE, 2012), and we strictly followed the manufacturer instructions. Before measuring anti-MV antibodies, sera were diluted 1:40 and calculated the relative immunity index (RI).The RI was defined as a coefficient that relate the optical density of controls (positive and negative) to that of the samples. RI values ranged from 1 to 10. Rabbits whose samples had an RI>2 were considered to be antibody positive (CIV TEST CUNI MIXOMATOSIS, HIPRA Laboratories, Girona, Spain). To measure anti-RHDV antibody levels, the INGEZIM kit for rabbits (INGENSASA Laboratories, Madrid, Spain) was used. Sera were screened using dilutions of 1:200, 1:400, 1:800, and 1:1600.

Samples presenting optical densities > 0.3 were considered to be antibody positive, since such antibody concentrations should be sufficient to confer protection against the disease (see Bertó-Moran et al., 2013). The test specificity and sensitivity were 83.1% and 98.5%, respectively, and there was 93% correspondence with the reference technique (OIE, 2012).

Rabbit abundance

Rabbit abundance was estimated by monthly pellet counts at fixed points (0.5 m^2) located within each enclosure, in the same habitat type (Cabezas and Moreno, 2007). A total of 30 fixed stations per enclosure were randomly arranged from May 2008 to April 2010. Every month we counted pellets and removed them later on for every counting station. As persistence of pellets in the field may be highly variable depending on the habitat type and/or season (Iborra and Lumaret, 1997; Palomares, 2001), we calculated the daily persistence of pellets according to the methodology described by Palomares (2001).

Data analysis

All statistical analyses were performed using R software. Specific data analyses in statistics procedures are explained in detail in each chapter.

CHAPTER 1

Multi-event capture-recapture modeling of host-pathogen dynamics among European rabbit populations exposed to myxoma and rabbit haemorrhagic disease viruses: common and heterogeneous patterns

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Abstract

Host–pathogen epidemiological processes are often unclear due both to their complexity and over-simplistic approaches used to quantify them. We applied a multi-event capture–recapture procedure on two years of data from three rabbit populations to test hypotheses about the effects on survival of, and the dynamics of host immunity to, both myxoma virus and rabbit haemorrhagic disease virus (MV and RHDV). Although the populations shared the same climatic and management conditions, MV and RHDV dynamics varied greatly among them; MV and RHDV seroprevalences were positively related to density in one population, but RHDV seroprevalence was negatively related to density in another. In addition, (i) juvenile survival was most often negatively related to seropositivity, (ii) RHDV seropositives never had considerably higher survival, and (iii) seroconversion to seropositivity was more likely than the reverse. We suggest seropositivity affects survival depending on trade-offs among antibody protection, immunosuppression and virus lethality. Negative effects of seropositivity might be greater on juveniles due to their immature immune system. Also, while RHDV directly affects survival through the haemorrhagic syndrome, MV lack of direct lethal effects means that interactions influencing survival are likely to be more complex. Multi-event modeling allowed us to quantify patterns of host–pathogen dynamics otherwise difficult to discern. Such an approach offers a promising tool to shed light on causative mechanisms.

Introduction

Emerging and re-emerging infectious diseases present one of the most pressing issues facing wild vertebrate populations in the 21st century (Dobson and Foufopoulos, 2001). However, relatively little is yet known about the exposure dynamics of infectious agents in their individual hosts, and what determines their impact on life-history traits such as survival (Benskin et al., 2009). A comprehensive understanding of the ecological processes influencing pathogen dynamics in natural host populations is of crucial importance to predicting both the dynamics of infectious diseases and the risks that they may entail for animal populations (Daszak et al., 2000; Chambert et al., 2012).

The European wild rabbit (*Oryctolagus cuniculus*) and the two main viral diseases that affect them (myxomatosis, and rabbit haemorrhagic disease) represent an important system for addressing wildlife eco-epidemiological issues. As we mentioned in the general introduction, the wild European rabbit is a species of great importance in the Mediterranean ecosystems but the emergence of myxomatosis and rabbit haemorrhagic disease (RHD) 60 years ago jeopardized seriously local populations.

In an attempt to prevent local extinction in its native range, and to avoid predator co-extinctions, the European rabbit has been made a conservation priority in both Spain and Portugal (Rogers, 1978; Moreno and Villafuerte, 1995; Palomares et al., 1995; Travaini et al., 1997).In addition the loss of rabbits, considered a pest species across most of their introduced range (Lees and Bell, 2008), has in some areas of the Iberian Peninsula caused large-scale economic loss and environmental degradation (e.g.(Virgós et al., 2007; Barrio et al., 2010)).

In spite of the ecological and economic relevance of these issues, disease surveillance still broadly uses simple counts of infected and uninfected animals,

although more accurate statistical tools that account for imperfect detection are now available (see Cooch et al., 2010 for a review of capture‐recapture modeling in epidemiology). A few studies have used simple Cormack‐Jolly‐Seber (CJS) capture‐recapture models to investigate the effects of myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) on rabbit population dynamics (e.g. Fordham et al., 2012; Guitton et al., 2008). As an extension of single-state (CJS) models, multi-state capture‐recapture models allow the direct estimation and testing of hypotheses about seroconversion rates and state-specific survival rates (Amason, 1973; Senar and Conroy, 2004; Faustino et al., 2004).

However, assessment of the infectious status of all captured individuals is often unfeasible, and some data rearrangement (e.g. data-censoring) is usually required for the application of multi-state models (reviewed in Conn and Cooch, 2009). To avoid these limitations, multi-event capture‐recapture models have been recently developed (Pradel, 2009) allowing uncertainty in state assessment to be modeled. Here we apply this novel approach to test hypotheses about the effects on survival of, and the dynamics of host immunity to, both MV and RHDV (which occur naturally in the study area).

As a general prediction, based on previous studies, we expected that seropositives for MV and RHDV have higher survival rates than seronegatives (e.g. Bruce et al., 2004; Calvete, 2006a; García-Bocanegra et al., 2010). In addition, we aimed to do the following: (i) estimate monthly survival rates dependent on antibody status, age (juveniles vs. adults) and sex, (ii) assess monthly seroconversion rates (from seropositive to seronegative, and vice versa) with respect to both diseases according to hosts' age and sex, (iii) quantify the dynamics of seroprevalence to each virus over

time, expressed as the seropositive probability, and (iv) examine relationships between population size and seroprevalence.

Materials and methods

Sampling

From May 2008 to April 2010, nine one-day live-trapping sessions were carried out at each enclosure. From a total of 6605 rabbits captured over the course of the study, we collected 1125 (17%) blood samples (1-2.5 mL) for serological analyses. There is more detail of the serology protocols that we employed in general materials and methods.

Multi-event capture–recapture analyses

Capture–recapture sessions were not synchronized among the three fenced areas (E1, E2 and E3), for logistic reasons. As a result, we ran separate analyses for each enclosure and disease agent (MV and RHDV). With these populations serving as breeding zones for restocking purposes, random samples of captured individuals were periodically removed during capture sessions (removals of individuals is coded in the data sets and does not affect estimation of parameters). Sample sizes for capture–recapture analyses (i.e. number of captures minus number of removals) were 2573, 1771 and 1400 for E1–E3 respectively. Numbers of animals captured per trapping session, divided by the surface of the trapped enclosure, were used as indices of rabbit density (Rouco et al., 2011) for each capture session and enclosure.

Goodness of fit

Since no goodness of fit (GOF) is available for multi-event models, for each population we used U-CARE 2.3.2 (Choquet et al., 2005) to test the fit of the Cormack–Jolly–Seber (CJS) model that accounted only for time variation in survival and capture probabilities. The CJS model is therefore more restrictive than those fitted for testing hypotheses that do account for the effect of serological status on recapture and transition probabilities. Hence, this approach is conservative given that if the CJS model adequately fits the data, then the multi-event models are also expected to fit.

For each population/GOF analysis, we defined four groups according to age at first capture (adult vs. juvenile) and gender. U-CARE allows testing for specific lack of fit due to a transience effect (i.e. a higher than expected presence of individuals showing up only once; test component 3.SR) and/or to trap-dependence (i.e. capture probability depending on the fact they were captured or not; test component 2.CT). When the overall (all the groups and components together) goodness-of-fit tests were significant, sources of extra-binomial variation were accounted for in the multi-event global models by including transience and/or trap dependence (according to the output of tests 3.SR and 2.CT on each specific group). Over-dispersion factors (c-hat) were then calculated as the ratio between the sum of $\chi 2$ values and degrees of freedom of the nonsignificant test components (Choquet et al., 2009).

For E1, the global goodness-of-fit test indicated lack of fit of the CJS model ($\chi 2 = 112.47$; d.f. = 87; P = 0.034), detecting “trap-happiness” among both adult males and females (P = 0.01 and < 0.01 respectively). For E2 and E3, there was no evidence of lack of fit ($\chi 2 = 73.53$; d.f. = 88; P = 0.87, and $\chi 2 = 73.53$; d.f. = 88; P = 0.8, respectively). We thus modeled trap-dependence among adults for E1 (see Pradel and

Sanz-aguilar, 2012) and Additional file 2 for details on the probabilistic framework used). Correction for over-dispersion (c-hat = 1) was not needed for any analysis.

Both survival and seroconversion rates have probably varied throughout the study period. However, because of limited sample sizes, and because our primary interest was in the net immunological effects on rabbit dynamics, we chose not to include a time effect on Survival and Seroconversion parameters.

Multi-event design

During each field session (excluding the fifth, for logistical reasons) a variable and random sample of captured individuals were blood-sampled (regardless of sex, age, and encounter history) and their MV and RHDV immunological statuses (seropositive or seronegative) were assessed. To account for uncertainty in state-assessment when an individual is not bled (sensu "partial observation"; Conn and Cooch, 2009) we used multi-event capture–recapture models (Pradel, 2005) in E-SURGE 1.8.5 (Choquet and Nogue, 2011).

Unlike traditional methods for handling partial information on states, like data censoring or the extra-state approach (Faustino et al., 2004), capture records in the multi-event framework are defined as events (i.e. reflecting the way the underlying biological states are observed in the field). It is therefore possible to define a specific event to record the capture of an individual whose state is unknown.

Here we considered four events (not seen, 0; seen, bled and assessed as seronegative, 1; seen, bled and assessed as seropositive, 2; seen and not bled, 3) and three possible states (dead, †; alive seronegative, SN; alive seropositive, SP). A slightly different set of states was used in models accounting for trap-dependence (details on the probabilistic framework are given in Additional file 2). Multi-event models include

three parameter types, Initial State (related to the probability of being in some specific state when first captured), Transition (related to the probability of transition between states) and Event (related to the probabilities of being re-sighted according to the event-mediated information on states). We decomposed Transition into two steps: Survival (the survival probability) and, conditional on still being alive, Seroconversion (the seroconversion probability). Event was decomposed into two steps: Capture (accounting for recapture probability) and, conditional on being captured, State Assignment (accounting for the probability the immunological status was assessed).

In this study, Initial State estimates the probability one first-captured individual is seropositive. Therefore, by assuming that the probability of first captured individuals being seropositive reflects the percentage of seropositive individuals in the population (but see Pradel, 2009 and Genovart et al., 2012 for a discussion on this), Initial State can be a proxy for the seroprevalence in each population.

We ran six analyses, one for each population–disease combination. We used QAICc values (Burnham and Anderson, 2002), to test for effects of immunity, age and sex on both rabbit survival and seroconversion rates (from seropositive to seronegative, and vice versa). Since populations were closed to immigration and emigration, survival rates referred to real survival rates (Lebreton et al., 1992). We assumed that time intervals were short enough that multiple transitions between serological states were unlikely to occur between two consecutive sessions and no bias was expected on seroconversion estimates (Zipkin et al., 2010).

In E-SURGE we marked the “uneven time intervals” option to allow monthly estimates of both survival and seroconversion probabilities even though intervals between capture sessions were not on a monthly basis. We also used the best model from each analysis to test the effect of population density on Initial State

(seroprevalence). For each analysis, we computed the significance and percentage of Initial State variation explained by density using analysis of deviance (ANODEV) (Skalski et al., 1993). This procedure compares the deviance and number of estimable parameters of three models identical except for the parameter of interest (Initial State in this case) which is: (i) constant, (ii) full-time dependent, or (iii) dependent on density.

Model selection

Based on preliminary model exploration, Initial State and State Assignment depended on, respectively, time and time-by-immunological status and were not further modeled. The other parameters of the global model accounted for these effects: (i) Survival on age-by-sex-by-immunological status, (ii) Seroconversion on sex-by-immunological status; Capture on sex plus age-by-immunological status by- time (in E1 also on trap-response).

For each population–disease combination, we first modeled recapture probabilities. The structure for recapture probabilities was then fixed as per the model with the lowest QAICc value, and Survival and Seroconversion probabilities were modeled independently. While we modeled Survival we kept the most parameterized structure for Seroconversion, and vice versa. For each parameter we considered a set of candidate models made of models nested to the global model.

To keep the number of tested models as low as possible (Burnham and Anderson, 2002), we only considered interactive effects for parameters whose time-variation was not modeled (i.e. Survival and Seroconversion). A final set of models combined the best structures for both Survival and Seroconversion (lowest QAICc when modeled independently) (Grosbois and Tavecchia, (2003) for a similar approach).

Hence we used this set of models to compute, for each parameter, model-averaged estimates from models lying within 2 Δ of the best model (Burnham and Anderson, 2002).

Results

Myxoma virus and survival

The relationship between MV seropositivity and survival varied among populations (Table 1, Figure 1). In E1, rabbit survival was variable among the age and sex classes but not between seropositives and seronegatives. In E2, MV-seropositive juveniles appeared to have lower survival rates than seronegatives (on average 25.1% lower, hereafter percentage differences refer to point estimates) but estimates were very imprecise; the same pattern was more evident among adult females (seropositive survival 9.6% lower) but the opposite trend was found among adult males (seropositive survival 6.8% higher). In E3, seropositives had higher survival rates than seronegatives in all age and sex classes (8.2% higher).

Table 1. Myxoma virus model-selection best models. (np, number of parameters; Dev, Deviance; QAICc, Quasi-Akaike Information Criterion corrected for over-dispersion; wi, Akaike weight (support of the current model with respect to the candidate set of models). Model notation: immun, the immunological status: for survival it means a different survival rate according to immunological status whereas for seroconversion it means that seroconversion rate from seropositive to seronegative is different than seroconversion from seronegative to seropositive; age, juveniles vs. adults; sex, females vs. males. For each analysis, only models accounting for more than 90% of cumulative Akaike weights are reported). *E1, E2 and E3 are the rabbit enclosures.*

Enclosure	Parameter	Model effects	np; Dev; QAICc; wi
E1	Survival	Age*sex	58; 6694.55; 6813.05; 0.66
		Age	56; 6700.78; 6815.10; 0.23
		Age*immun	58; 6699.49; 6817.99; 0.06
	Seroconversion	Sex*immun	62; 6691.23; 6818.08; 0.92
E2	Survival	Age*sex*immun	53; 4733.87; 4053.88; 0.88
		Age*immun	49; 4750.48; 4059.29; 0.06
	Seroconversion	Sex*immun	53; 4733.87; 4053.88; 1
E3	Survival	Immun	48; 3664.89; 3764.20; 0.69
		Age*sex*immun	54; 3654.65; 3766.85; 0.18
		Sex*immun	50; 3664.83; 3768.43; 0.08
	Seroconversion	Immun	52; 3656.86; 3764.76; 0.71
		Sex*immun	54; 3654.65; 3766.85; 0.25

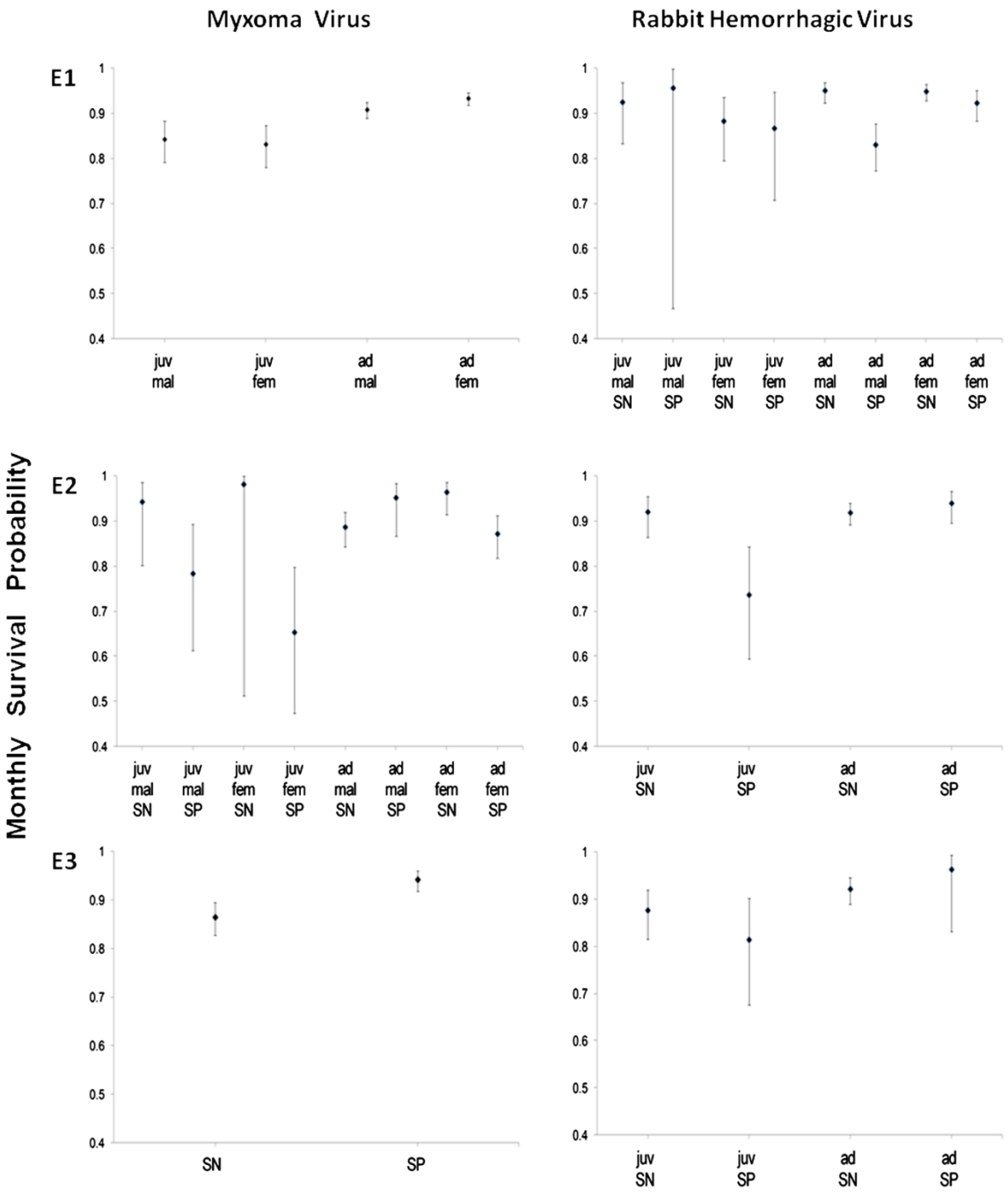

Figure 1. Average monthly survival probability according to serological status (to MV and RHDV), age and gender in the three enclosures (E1, E2, and E3). Only estimates related to effects selected in models with lowest QAICc are shown (e.g. if sex effect on survival was found to be negligible by model selection, one common estimate is shown for both males and females). Estimates with 95% confidence intervals are shown. Notation: juv, juvenile; SN, seronegative; SP, seropositive; mal, males; fem, females.

Rabbit haemorrhagic disease virus and survival

The relationship between RHDV seropositivity and survival also varied among populations (Table 2, Figure 1). However, there was a general pattern of seropositives tending to have lower survival than seronegatives. In E1, there was no clear relationship between RHDV seropositivity and either juvenile or adult female survival, whereas it was related to lower survival (12.6%) in adult males. In E2, seropositivity was related to lower juvenile survival (20%), but no relationship was observed for adults. In E3, seropositive juveniles appeared to survive less (7.1%) than seronegatives, whereas no clear effect was found for adults.

Table 2. Rabbit haemorrhagic disease virus model-selection best models. E1, E2 and E3 are the rabbit enclosures (See Table 1 for abbreviation notations).

Enclosure	Parameter	Model effects	np; Dev; QAICc; wi
E1	Survival	Age*sex*immun	83; 6518.66; 6689.61; 0.73
		Sex*immun	79; 6530.35; 6692.83; 0.15
		Immun	77; 6535.99; 6694.24; 0.07
	Seroconversion	Immun	81; 6519.89; 6686.59; 0.74
		Sex*immun	83; 6518.66; 6689.61; 0.16
E2	Survival	Age*immun	49; 4724.47; 4037.61; 0.62
		Age*sex*immun	53; 4718.07; 4040.71; 0.13
		Age	47; 4733.35; 4040.81; 0.13
		Age*sex	49; 4729.07; 4041.44; 0.09
	Seroconversion	Sex*immun	53; 4718.07; 4040.71; 0.82
		Sex	51; 4726.87; 4043.82; 0.17
E3	Survival	Age*immun	42; 3830.64; 3917.17; 0.42
		Age	40; 3835.32; 3917.62; 0.34
		Immun	40; 3837.85; 3920.15; 0.09
		Age*sex	42; 3834.10; 3920.63; 0.07
	Seroconversion	Sex*immun	46; 3827.61; 3922.65; 0.54
		Immun	44; 3832.35; 3923.13; 0.43

Myxoma virus seroconversion

Overall, the probability of becoming MV seropositive was higher than that of becoming seronegative (Table 1, Figure 2). In E1, both males and females became seropositive at a faster rate than the reverse; for females the probability of becoming seronegative was null suggesting that female rabbits once seropositive remain so. Males appeared to become seropositive at a faster rate than females. In E2, females became seropositive at a faster rate than the reverse, and faster than males. In E3, the probability of becoming seropositive was the same for both genders, and the reverse was null.

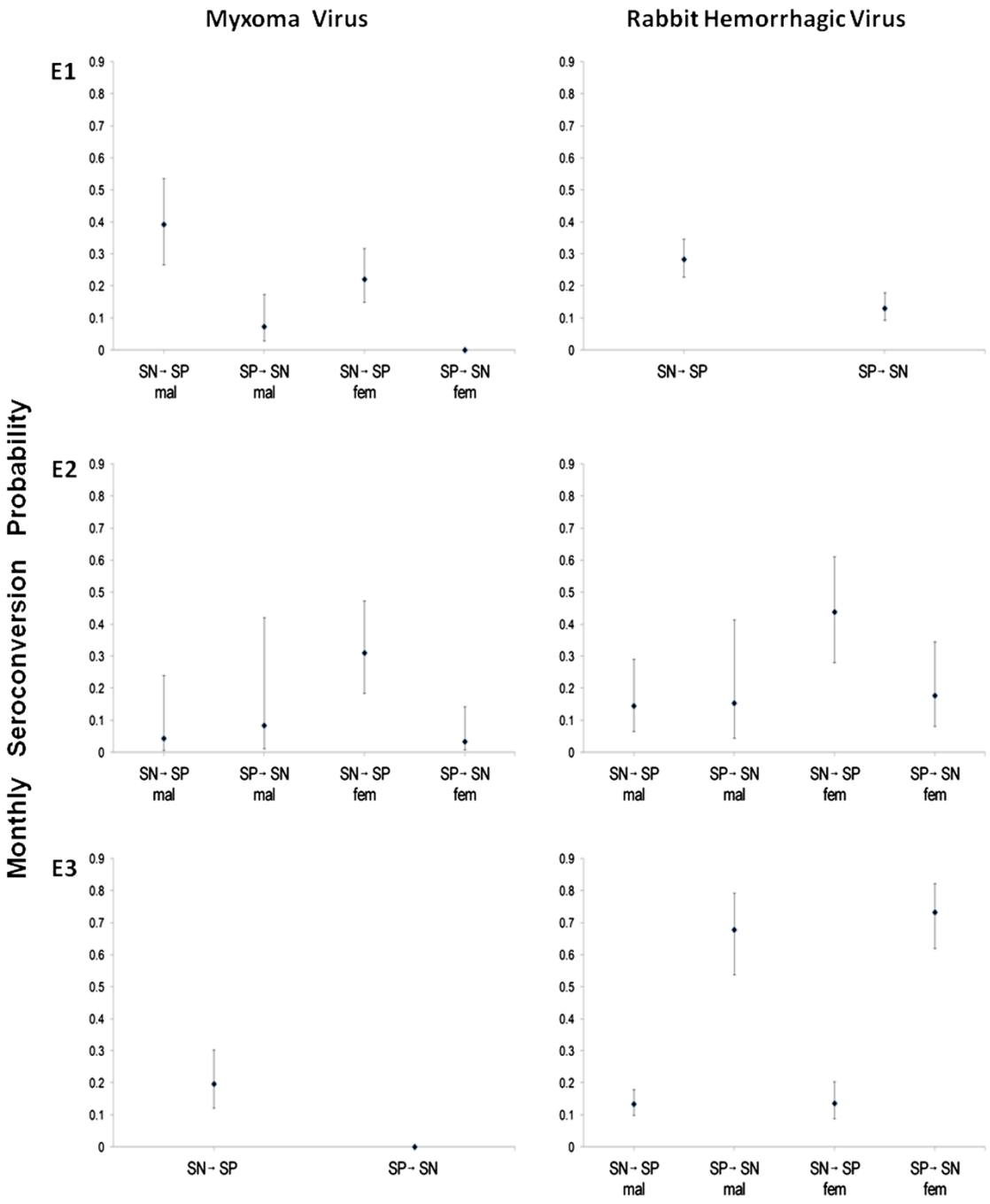

Figure 2. Myxoma virus and rabbit haemorrhagic disease virus seroconversion rates in the three enclosures (E1, E2, and E3). Estimates with 95% confidence intervals are shown. Notation: SN→SP, average monthly rate at which individuals change their immunological status from seronegative to seropositive; SP→SN, from seropositive to seronegative; mal, males; fem, females.

Rabbit haemorrhagic disease virus seroconversion

We found very different patterns of RHDV seroconversion among enclosures (Table 2, Figure 2). In E1, no difference was observed among genders, with all rabbits becoming seropositive at a faster rate than the reverse. In E2, there were no consistent differences in seroconversion rates of males, while females became seropositive at a higher rate than the reverse (and faster than males). In contrast, in E3 both sexes became seropositive at an unusually high rate, and faster than they became seronegative (from model without sex effect: respectively, 0.72, SE: 0.05; 0.14, SE: 0.02).

Seroprevalence and density

Seroprevalence to MV and RHDV varied across time in all populations (Table 3), but high levels of uncertainty on several estimates made it difficult to discern time-trends. Population size in the three enclosures followed similar patterns, being lower during the winter and higher during the spring.

The three populations reached maximum peak densities at the same time in May 2010, while minimum densities occurred at different times. Average rabbit densities (no. /hectare) per enclosure (± 95% CI) over the whole study period were 79.6 ± 53.1, 49.9 ± 31.4, and 52.5 ± 38.5 (for E1–E3 respectively). We observed a marginally significant positive relationship between rabbit density and MV seroprevalence for E1 (slope on logit scale: 1.73; SE: 0.3; P: 0.07; 66% variation explained), and a negative such relationship for E3 (slope on logit scale: −1.13; SE: 0.26; P: 0.06; 71% variation explained).

We also observed a marginally significant positive relationship between density and RHDV seroprevalence in E1 (slope on logit scale: 1.41; SE: 0.17; P: 0.06; 73% variation explained).

Table 3. Effect of rabbit density on Initial State (seroprevalence to myxoma virus and rabbit haemorrhagic disdease). From left to right: the Fisher–Snedecor statistic (F1,7 for Initial State and F1,6 for the others), P-value (in bold if < 0.1), and R2. All statistics were computed following the ANODEV procedure. MV, myxoma virus; RHD, rabbit haemorrhagic disease virus; Prev, prevalence.
E1, E2 and E3 are the rabbit enclosures.

	MV-Prev	**RHD-Prev**
E1	4.65; 0.07; 0.66	5.11; 0.06; 0.73
E2	0.25; 0.63; 0.03	0.80; 0.4; 0.11
E3	5.01; 0.06; 0.71	2.94; 0.13; 0.42

Recapture probability

Recapture rates varied through time in all analyses (average estimates ± SD from best models of MV analyses: E1, 0.61 ± 0.22; E2, 0.48 ± 0.19; E3, 0.28 ± 0.19).We found either sex, age, or immunological status affected the probability of being recaptured (in E1 trap happiness was also confirmed throughout model selection). However, very heterogeneous causal effects on recapture probabilities were found among and within the rabbit populations.

Discussion

Our study confirms that rabbit populations respond very differently in terms of host survival and epidemiological dynamics when exposed to MV and RHDV, even if geographically close to each other (Parkes et al., 2002; Fouchet et al., 2008). Multiple interacting causes likely explain this heterogeneity. However, some general patterns were observed in our study: (i) RHDV seropositivity was never related to increased rabbit survival; (ii) seropositivity to both MV and RHDV was negatively related to survival more frequently for juveniles than for adults; and (iii) once MV seropositive, rabbits rarely lost such status.

Survival

Our results do not conform to the overall published pattern of MV and RHDV seropositive rabbits always having higher survival rates than seronegatives (Bruce et al., 2004; Calvete, 2006a; García-Bocanegra et al., 2010). Only in one case, MV seropositives had considerably higher survival rates than seronegatives. However, it should be noted that our populations were in semi-natural captive conditions allowing high densities, which greatly exceeded wild population densities (e.g. c. 3 rabbits/ha for the central Iberian Peninsula; (Fernández de Simón et al., 2011)). Therefore our results could be extrapolated to high density scenarios in the wild (e.g. Moller et al., 1997; Mutze et al., 2010).

Seropositivity to MV and RHDV could potentially confer both advantages and disadvantages to rabbits. While it may confer higher immunity to these pathogens, several authors have recognized that (for MV at least) seropositivity has an immunosuppressive effect that may cause higher rates of co-infection with other

pathogens (Boag, 1988; Cattadori et al., 2007; Cattadori et al., 2008). With the target organ of RHDV (the liver) being part of the immune system, RHDV may also cause an immunosuppressive syndrome in addition to reduced survival due to haemorrhagic effects (Mitro and Krauss, 1993; Ferreira et al., 2004; Ferreira et al., 2006).

Rabbits seropositive to either virus are thus likely to be more susceptible to damage from other infections. A shifting balance of advantages and disadvantages is thus one explanation for contrasting patterns, and is likely influenced by interactions with other environmental, epidemiologic and individual factors. For example, in our population E3 rabbits were in a better physiological status than in the others as a result of availability of higher water quality (e.g. in streams, manuscript in preparation). We suggest that this reduced the negative effect of the MV-seropositive-related immunosuppression, explaining why they had higher survival rates than seronegatives.

As another example, RHDV seropositivity never being associated with higher survival rates could indicate that the negative effects of the haemorrhagic syndrome outweighed any beneficial effects. Finally, seropositive juveniles tending to survive less than seronegatives may be explained by the immaturity of the juvenile immune system with full immunity not yet being fully acquired. In light of this, it is possible that negative influences such as immunosuppression and the haemorrhagic syndrome of RHDV had greater influence on juveniles than adults.

Seroconversion

With ongoing persistence 50 and 30 years after the arrival of myxomatosis and RHD respectively on the Iberian Peninsula (Calvete et al., 2002), it is reasonable to classify the viruses as endemic. Accordingly, rabbits should gain MV and RHD antibodies as fast as or faster than they lose them. Seroconversion to seropositive status

tending to occur at a faster rate than the reverse in our study (and also Calvete et al., 2002 and García-Bocanegra et al., 2010) supports this expectation.

However, this was not the case for RHDV in E3, where seroconversion to seronegative status occurred at a higher rate. We suggest this result does not disprove the endemic disease behavior hypothesized above, but is driven by asynchrony between population and virus dynamics. In fact, RHDV was found in this population over the 7 years of monitoring (SM, unpublished data), but the population had a delayed breeding season that, differently from in the other enclosures, occurred after the RHD outbreak.

Thus, in E3 the naïve kittens would have lost their maternal antibodies after 2 months (Calvete et al., 2004b) and, since they were not exposed to RHDV, a large number of seronegative adults (many of them born the spring just before) would have occurred in the next autumn. This would result in a large number of seronegatives over the study period and explain this seemingly inconsistent result. It should also be noted that, in contrast to general belief (Kerr et al., 2010), the average monthly probability of losing antibodies was not necessarily null for either RHDV or MV. This indicates that rabbits can lose immunity to these diseases (albeit with a very low probability).

The effect of sex on the rate at which individuals became seropositive varying among populations further illustrates the varying behavior of these diseases across individuals and populations (Parkes et al., 2002; Fouchet et al., 2008).

Antibody prevalence and density

In general, seroprevalence did not follow a consistent pattern either within or among populations for either disease. This was in agreement with previous findings from some authors, stating these diseases behave very differently among populations (Parkes et al., 2002; Fouchet et al., 2008), but contrasts with a study in the Canary

Islands (Foronda et al., 2005) where they found no difference in RHDV prevalence across four neighboring geographic zones.

Host infection by both viruses also occurs by means of contact with an infected individual. Population size is thus recognized as an important factor promoting MV and RHDV dynamics (Fouchet et al., 2008; Cooke and Fenner, 2002; Calvete, 2006b; Cotilla et al., 2010). However, even though in some cases a great amount of variation in seroprevalence appeared to be explained by density, in no case did we find this hypothesis was strongly supported (P-values were marginally significant at the 0.05 level).

The pattern of increasing prevalence with population size observed in E1 for both MV and RHDV was unsurprising. However, we observed no such relationships in E2 and a negative relationship between MV prevalence and population size in E3. This last result may have been caused by a correlation between decreasing density due to gradual habitat degradation (authors personal observation) leading to individuals under nutritional stress being more susceptible to infection.

Conclusion

This is the first multi-event study focusing on MV and RHDV host–pathogen dynamics in rabbit populations. We found that while MV seropositivity had either a positive or negative effect on survival that was likely dependent on interaction with other factors (e.g. physiological condition), the haemorrhagic syndrome caused by RHDV led seropositive rabbits to suffer higher mortality rates.

Our study highlights that the host–pathogen dynamics of these viruses are highly variable among populations even when these share similar management and climatic

conditions. These findings have important implications for rabbit population management, particularly where their scarcity could compromise ecosystem conservation.

Additional well-defined capture–recapture analyses may shed further light on the still many obscure mechanisms driving host–pathogen dynamics (e.g. Lachish et al., 2011a; Lachish et al., 2011b).

CHAPTER 2

Coccidian and nematode infections influence prevalence of antibody to myxoma and rabbit haemorrhagic disease viruses in European rabbits

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Abstract

The interaction among several parasites in European rabbits (*Oryctolagus cuniculus*) is crucial to host fitness and to the epidemiology of myxomatosis and rabbit haemorrhagic disease. These diseases have caused significant reductions in rabbit populations on the Iberian Peninsula. Most studies have focused on the epidemiology and pathogenesis of these viruses individually, and little is known about interactions between these viruses and other parasites. Taking advantage of an experimental restocking program in Spain, the effects of coccidian and nematode infections on the probability of having detectable antibody to myxoma and rabbit haemorrhagic disease viruses were tested in European wild rabbits. For 14 months, we monitored rabbit abundance and parasite loads (coccidia and nematodes) in three reintroduced rabbit populations. While coccidian and nematode loads explained seasonal seroprevalences to myxoma virus, the pattern was less clear for rabbit haemorrhagic disease. Contrary to expectations, seroprevalence to myxoma virus was inversely proportional to coccidian load, while nematode load seemed to play a minor role. These results have implications for viral disease epidemiology and for disease management intended to increase rabbit populations in areas where they are important for ecosystem conservation.

Introduction

The arrival of two viral diseases, myxomatosis in the 1950s and rabbit haemorrhagic disease (RHD) in the 1980s, was the primary cause of a significant reduction in the European rabbit (*Oryctolagus cuniculus*) population on the Iberian Peninsula (Delibes-Mateos et al., 2009). Because the European rabbit is the staple prey for more than 30 predators in Spain, this population crash caused a significant perturbation to the ecosystem (Delibes- Mateos et al., 2008). Populations of some predators, including the Iberian lynx (*Lynx pardinus*) and the Iberian Imperial Eagle (*Aquila adalberti*), are now endangered (Ferrer and Negro, 2004); therefore, the recovery of wild rabbit populations has become a major goal of nature conservation in Spain. Although management techniques have been applied (Moreno and Villafuerte, 1995; Calvete et al., 2004c; Cabezas and Moreno, 2007; Rouco et al., 2008, 2011), they have been only partly successful in mitigating viral diseases, and annual outbreaks of myxomatosis and RHD cause high mortality in European Mediterranean ecosystems.

Most studies have focused on the epidemiology and pathogenesis of these diseases separately (Calvete et al., 2002), and the potential role of coinfection has been so far neglected. Studies have shown different coinfection relationships between myxoma virus (MV) and parasite infections (Boag, 1988; Boag et al., 2001; Lello et al., 2005).

Two long-term monitoring studies (Cattadori et al., 2007, 2008) have led to two important conclusions concerning the dynamics of coinfection under natural conditions. Rabbits infected with MV are more susceptible to nematode infection, and rabbits with existing nematode infestations suffer longer MV infections. The immune response plays a key role in these interactions. It is generally accepted that after infection, naïve T

helper (Th) cells begin to differentiate into Th1 and Th2cells, each characterized by a specific type of interleukin (IL). Thus, the response triggered by MV (as a microparasite) biases the system toward Th1, but immune defense against nematodes (macroparasites) requires a Th2 response (Cox, 2001; Pedersen and Fenton, 2006). An experimental study showed that if both pathways occur simultaneously, the relaxation of the immune response provokes higher mortality (Kerr et al., 2004). Thus, the interactions between the two viral diseases and other parasites remain unclear, and there is little information about the ways in which other factors, such as host age, sex, body condition, reproductive stage, season, or abundance, influence these pathogen interactions.

We tested whether the loads of microparasites (belonging to the family Eimeriidae, subclass Coccidiasina) and macroparasites (gastrointestinal nematodes) influence the ability of rabbits to generate an appropriate immunologic response against MV and rabbit haemorrhagic disease virus (RHDV). Because susceptibility to a given pathogen would be affected by the ongoing cytokine response due to a preexisting infection (Graham et al., 2007), we tested two predictions: (1) Populations with higher nematode loads will present lower seroprevalences to both viruses, because gastrointestinal nematodes would polarize the immune system toward Th2, and (2) populations with higher coccidian loads will have higher seroprevalences, because coccidian and viruses are regulated by the same Th1 pathway (Yun et al., 2000; Pedersen and Fenton, 2006).

Materials and methods

Sampling

From January 2009 to April 2010, five live-trapping sessions were conducted in each enclosure. From 6605 rabbits originally captured, we selected and transported 563 adults (253 females and 310 males) to a field laboratory within each enclosure to take blood samples. Blood samples were used to obtain anti-MV and anti-RHDV serum antibody concentrations. Also rabbit abundance was calculated by using pellet counts. For further details of serology and rabbit abundance protocols see general materials and methods.

Parasitologic analyses

To analyze the load of coccidia and nematodes within each enclosure, 20 samples with five fresh pellets each were randomly collected each month (Coudert et al., 2000). To avoid samples from juveniles, only pellets .6 mm in diameter were collected (Rouco et al., 2012). Fecal egg and oocyst counts were determined by the modified McMaster technique (Raynaud, 1970), and results are expressed as oocysts per gram (opc) of faeces for coccidia or eggs per gram (epg) of faeces for nematodes. No other group of parasites was found.

Data analyses

To evaluate the predictions, a model selection approach was followed performing generalized linear mixed models (GLMMs) using a binomial distributed error with a logistic link function.

The probability of being antibody positive (categorical variable: antibody-negative=0, antibody-positive=1) for both MV and RHDV (as response variables) was explained by the single effect and the two-way interactions of the following explanatory variables: nematode load (epg), coccidian load (opg), rabbit abundance (pellets/m2), sex, month, and body condition.

Body condition was obtained with residual values after performing a simple linear regression between log-transformed body weight and tarsus length (Cabezas et al., 2006). Because animals were systematically sampled from the same enclosures, the study site was included as a random factor in our analyses. Because all samples came from adult animals, age was excluded from the analysis.

Model selection was performed following a theoretic information approach based on Akaike's information criterion (AIC; Anderson et al., 2001; Johnson and Omland, 2004). Briefly, for each candidate model, the AIC was estimated by selecting the model with the lowest value; we then ranked the remaining competing models according to their AIC value and subsequently estimated their Akaike differences (Δi) with respect to the best model (lowest AIC). Subsequently, the Akaike weights (*wi*) were estimated, defined as the relative probability of each model to be the best one among those being compared (Anderson et al., 2001). The absence of a pattern in residual values of the selected models was confirmed (Zuur et al., 2009). In order to discuss the effect size, the explained deviance of the best model and the pure deviance of each variable were calculated (Zuur et al., 2007). Statistical analyses were done using R version 2.12.2. Specifically, GLMMs were implemented using the ''lme4'' package, R package version 0.999375- 35 (R Development Core Team, University of Auckland, Auckland, New Zealand).

Results

Myxoma virus seroprevalence peaked seasonally in February and October and was similar in the three enclosures (Fig. 1). For RHDV, enclosures 2 and 3 (but not enclosure 1) had annual maxima in April and October (Fig. 1). Coccidian and nematode loads had specific monthly patterns with maximum values in April (Fig. 1).

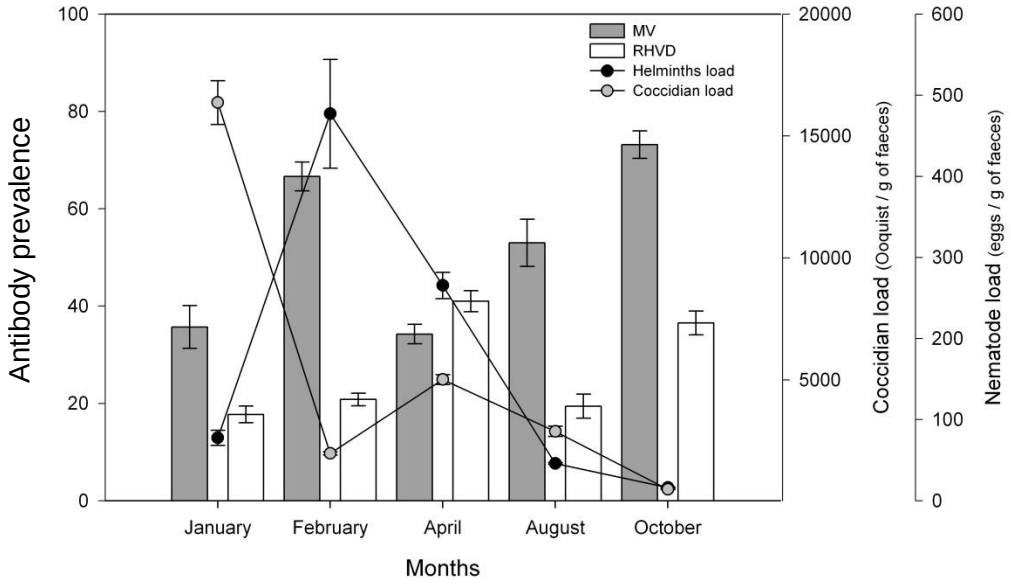

Figure 1. Average monthly seroprevalence to myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) and coccidian and nematode (helminth) load in three European rabbit (Oryctolagus cuniculus) populations.

The model selection showed that prevalence of antibody against MV was influenced by both coccidian and nematode loads in each enclosure (Table 1 and Fig. 2). In the best model for MV (*wi*Mo+Coccidian load+Nematode load, Table 1), 16% of the observed variability in the probability of being antibody-positive was explained by month (βMo = -20.04, standard error [SE] = 0.008, *t*-value = -5.4) and also for the coccidian ($\beta Coccidian$ = -20.49, SE = 0.05, *t* = 29.23) and nematode ($\beta Nematode$ = -

0.22, SE = 0.03, *t* = -6.22) loads in each enclosure. However, coccidian and nematode loads shared 1.7% of the explained deviance (they were positively correlated, β = 0.25, SE = 0.03, *t* = 6.8, R^2 =7.7%). Correcting for this, the pure effect of coccidian load was about 4%, and that of nematode load was 1.4%. The influence of parasites persisted after correcting for season, being less intense in winter-spring (e.g., January, February, and April, β*Coccidian* = -20.37, SE = 0.06, *t* = -5.7, and β*Nematodes* = -0.94, SE =0.18, *t* = -5.2, explaining for both factors 14% of the observed variability in MV antibody prevalence) than in summer- autumn (e.g., August and October, β*Coccidian* = -0.48, SE = 0.07, *t* = -6.5, and β*Nematodes* = -0.36, SE = 0.14, *t* = -2.46, explaining for both factors 15.8% of the observed variability in MV antibody prevalence). Other factors, such as strict rabbit abundance (β*Abundance* = 0.15, SE = 0.05, *t* = 3.05, explaining 1.1% of the observed variability in MV antibody prevalence) or body condition (β*Body condition* = 0.55, SE = 0.34, *t* = 1.5, explained deviance = 0.2%), had a slight positive effect on the probability of being antibody-positive. Sex had no effect on the rate of MV antibody prevalence (β*Sex = 0*.04, SE = 0.04, *t* = 0.9, deviance explained = 0.07%).

There was no clear effect of parasite load, rabbit abundance, body condition, or sex for RHDV antibody prevalence, since the null model (Mo) was placed at 0.09 points from the best model, and the AIC weights for both models were similar (Table 1).

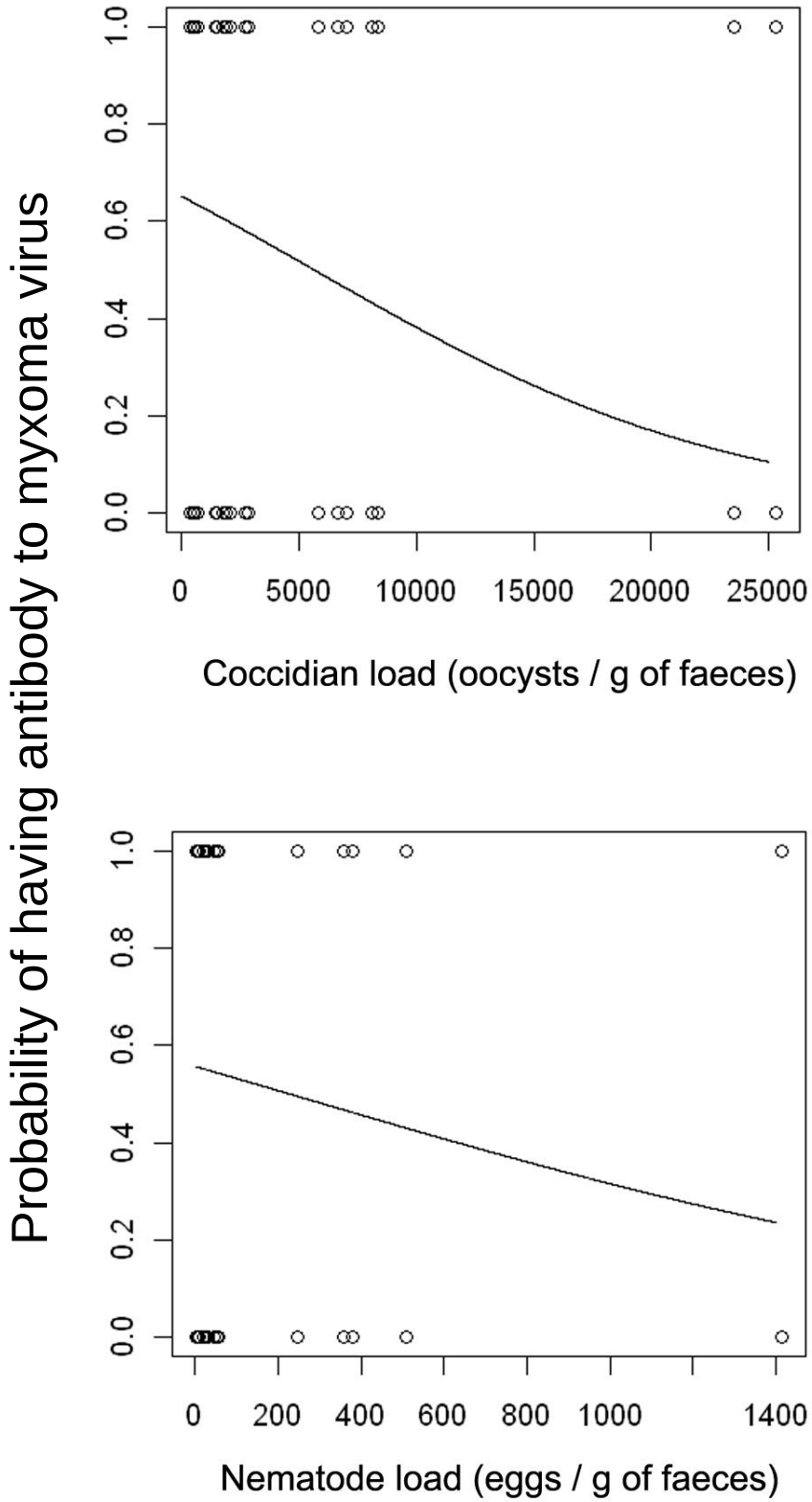

Figure 2. Effect of coccidian and nematode load on probability of having detectable antibody to myxoma virus in three European rabbit (Oryctolagus cuniculus) populations.

Table 1. Model selection for probability of having detectable antibody to myxoma and rabbit haemorrhagic disease (RHD) viruses in a sample of 563 European wild rabbits (Oryctolagus cuniculus) in the southwestern Iberian Peninsula. Models with substantial support for being the best model are represented in bold. [a]

Biological models	*K*	AIC	Δ*i*	*wi*
Myxoma virus				
Mo+coccidian load+nematode load	6	707.39	0	0.99
Coccidian load+nematode load	5	726.24	18.80	<0.001
Mo	3	810.98	103.48	<0.001
RHD virus				
Abundance+coccidian load+nematode load	6	706.97	0	0.30
Mo	3	707.06	0.09	0.29
Helminth load	4	708.65	1.68	0.13
Coccidian load+nematode load	5	709.54	2.57	0.09
Body condition+nematode load	5	710.62	3.65	0.05
Body condition	4	711.03	4.05	0.04
Abundance+coccidian load	5	712.48	5.51	0.02
Abundance+nematode load	5	713.23	6.26	0.01
Abundance	4	713.25	6.28	0.01
Mo+nematode load	5	713.33	6.36	0.01
Coccidian load	4	713.38	6.41	0.01
Sex	4	714.21	7.24	<0.001
Body condition+coccidian load	5	715.73	8.76	<0.001

[a]K = *number of parameters including intercept;* AIC = A*kaike Information Criterion;* ΔI = *difference of AIC with respect to the best model*; *wi0 Akaike weigth;* Mo=*null model only with the constant term.*

Discussion

Parasite load was a clear explanatory factor for prevalence of antibody to MV but not RHDV in European hares. However, contrary to our initial prediction, in the three populations, higher seroprevalence to MV occurred at low coccidian load.

Before discussing our results, a methodological limitation should be considered. Data on antibody prevalence come from individual rabbits, while data on coccidian and nematode load represent the entire population. It was impossible to obtain both blood and fresh pellet samples from the same captured rabbits, as would have been ideal. However, both kinds of samples were taken simultaneously and always represent the same adult population. Thus, our results are likely to be a reliable approximation that could be tested in the laboratory.

Coccidian infection is one of the main predisposing factors for intestinal enteropathies and can cause higher fatality rates than nematodes (Varga, 1982; Hobbs et al., 1999a), especially as animals that have recovered from coccidiosis are immunocompromised (Yun et al., 2000). Nevertheless, in our study, coccidian infections were more common than nematode infections in the wild, as others have also found (Peeters et al., 1981). Thus, it is likely that coccidian load has played a key role in generating an immunologic response, since there is higher probability of both pathogens (MV and coccidian) occurring in the same animal.

Contrary to our initial hypothesis, populations with lower coccidian load had higher prevalences of antibody to MV. These patterns of coinfection (e.g., nematode-coccidian-MV) could be partially due to an immunosuppressive effect of MV by decreasing circulating Th cells, and the remaining Th cells polarize the system to Th2 (Jeklova et al., 2008). Thus, the immune response against nematodes is not completely

disrupted by the response to MV (Cattadori et al., 2008), but dealing with coccidia and MV simultaneously requires a high Th1 response that the immune system is unable to produce. In spite of this, the trade-off of the Th1/Th2 response must not be the only mechanism of the immune system to deal with coinfections (Cox, 2001). Other ecologic and environmental factors likely play an important role that was not considered in this study. In any case, it seems to be clear that coccidian control could play a key role in combating rabbit diseases (Peeters et al., 1984).

In the context of RHDV coinfection, our model showed no relationship between the explanatory variables and the ability to develop RHDV antibody. Differences in the pathogenesis of both viruses could explain these results. The way our explanatory variables work is easier to understand in an immunosuppressive virus (MV), and, therefore, different variables should be considered to understand the ability to mount an antibody response to RHDV. We were surprised that the model did not select rabbit abundance as an influencing variable because several authors have reported a strong relationship between this variable and MV antibody prevalence (Calvete et al., 2002; Fouchet et al., 2008). Moreover, the transmission of MV is density dependent; the virus disappears after an epidemic in smaller populations but becomes endemic in large ones (Fouchet et al., 2008). Because antibodies against MV are maintained in rabbits for life (Fouchet et al., 2008), and the virus is widespread in the wild in southern Spain (García-Bocanegra et al., 2010), MV antibody prevalence is higher in dense populations. The absence in our analysis of data from juvenile rabbits, responsible for the overall seroconversion rates, was probably the cause of these results.

Our results agree with those of Blasco et al. (1996), who found that the highest prevalences of parasitization occurred during the breeding season, preceding peak population abundance; thus, a delay between coccidian and nematode levels and rabbit

abundance occurs. For this reason, the highest MV seroconversion rates would occur in rabbit populations that have lower parasite loads and higher densities. Several authors have demonstrated that rabbit abundance plays a similar role in the epizootiology of RHDV and MV (Calvete et al., 2002; Cooke, 2002). Yet, we did not find a clear relationship between population abundance and RHDV antibody prevalence.

Further studies are needed to understand this absence of a relationship. Although more information is required, antibody prevalence to MV clearly depended heavily on coccidian load. High seroprevalence occurred when coccidian load was low, while nematode load played a minor role in this process. Our results have implications not only for the viral disease epizootiology, but ultimately, for disease management aimed to increase rabbit populations in areas where the rabbit is a keystone species for ecosystem conservation.

CHAPTER 3

Effects of myxoma virus and rabbit haemorrhagic disease virus on the physiological condition of wild European rabbits: Is blood biochemistry a useful monitoring tool?

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Abstract

Myxomatosis and rabbit haemorrhagic disease (RHD) are the major viral diseases that affect the wild European rabbit (*Oryctolagus cuniculus*). These diseases arrived in Europe within the last decades and have caused wild rabbit populations to decline dramatically. Both viruses are currently considered to be endemic in the Iberian Peninsula; periodic outbreaks that strongly impact wild populations regularly occur. Myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) alter the physiology of infected rabbits, resulting in physical deterioration. Consequently, the persistence and viability of natural populations are affected. The main goal of our study was to determine if blood biochemistry is correlated with serostatus in wild European rabbits. We carried out seven live-trapping sessions in three wild rabbit populations over a two-year period. Blood samples were collected to measure anti-MV and anti-RHDV antibody concentrations and to measure biochemical parameters related to organ function, protein metabolism, and nutritional status. Overall, we found no significant relationships between rabbit serostatus and biochemistry. Our main result was that rabbits that were seropositive for both MV and RHDV had low gamma glutamyltransferase concentrations. Given the robustness of our analyses, the lack of significant relationships may indicate that the biochemical parameters measured are poor proxies for serostatus. Another explanation is that wild rabbits might be producing attenuated physiological responses to these viruses because the latter are now enzootic in the study area.

Introduction

Diseases can represent major threats for wild animal populations because they can lead to decline and extinction (Viggers et al., 1993; Woodroffe, 1999; Mörner et al., 2002). In fact, acquiring a better understanding of diseases and pathogens is a crucial but challenging task in wildlife conservation efforts (Deem et al., 2001). In ecosystems, host-pathogen relationships help shape patterns of species distribution and persistence (Dobson and Hudson, 1986; Thomas et al., 2005; Collinge et al., 2006; Hudson et al., 2006). Even though most previous studies have focused on one-host, one-pathogen systems, such dynamics are actually rare in nature. Individual hosts are often co-infected by multiple pathogens, which interact in complex ways with each other (Pedersen and Fenton, 2007). Therefore, studying the mechanisms underlying these interactions is of primary importance if we wish to predict how pathogens will affect host physiology and if we want to effectively control target and non-target parasite species.

Despite its relevance for wildlife conservation and management, the physiology of wild species is rarely studied because physiological parameters are difficult to quantify. Furthermore, it is challenging to combine physiological information with other data, such as antibody concentrations, at the population level. By incorporating indices of host physiological condition into population surveillance and monitoring efforts, we will gain deeper insight into the range of host responses and pathogen effects. Such tools could reveal the status of major pathogens within wild animal populations and provide a snapshot of a given animal's physiological state; consequently, they would serve as more straightforward means of assessing population condition. In this study, we used the wild European rabbit

(*Oryctolagus cuniculus*) and its two main viral diseases, myxomatosis and rabbit haemorrhagic disease (RHD), as a model system.

At present, myxomatosis and RHD are endemic diseases in the Iberian Peninsula; they cause periodic outbreaks that significantly impact natural populations (Calvete et al., 2002). Outbreak patterns suggest that these viruses are in continuous recirculation and are largely associated with the breeding season; myxomatosis outbreaks occur predominantly in summer and autumn, while RHD outbreaks occur in winter and spring. It also appears that the viruses remain in the same areas from one year to the next (Calvete et al., 2002). Factors such as breeding season length and timing, host population size, vector abundance, and environmental conditions have major effects on the duration and potential impact of the epizootics and, ultimately, on virus persistence within populations (Fouchet et al., 2008). We currently have a good grasp of the epidemiology and pathology of myxomatosis and RHD, topics that are discussed extensively in the literature (e.g., Fenner et al., 1953; Liu, 1984; Xu, 1991; Cooke, 2002; Calvete et al., 2002; Stanford et., al 2007; Abrantes et al., 2012). Myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) dramatically alter the physiology of infected rabbits. These alterations result in the deterioration of physical health, which we will hereafter refer to as physiological condition (Kerr and Donnelly, 2013). In general, an individual's physiological condition is negatively correlated with the degree of infection burden but positively correlated with immune function (Chandra and Newberne, 1997; Gershwin et al., 1985; Møller et al., 1998). Therefore, rabbits in poor physiological condition may also be more likely to become infected (Nelson and Demas, 1996; Tompkins and Begon, 1999; Beldomenico et al., 2008).

There is a need for straightforward, reliable methods for assessing the physiological condition of wild rabbits; past studies suggest that blood biochemistry could be helpful in this regard (Franzmann and Shwartz, 1988; Hellgren et al., 1989; Schroeder, 1987; Hellgren et al.,

1993; Milner et al., 2003). Moreover, as compared to more conventional measures, biochemical parameters are highly sensitive, meaning they change to reflect an individual's physiological state in a matter of minutes. Consequently, the use of blood biochemistry may make it possible to identify rabbits experiencing extreme stress in general (Milner et al., 2003).

In this study we monitored blood chemistry and MV and RHDV serostatus in wild populations of the European rabbits. Our main objectives were the following 1) to assess the usefulness of biochemical parameters as predictors of an individual's physiological condition; 2) to determine if a relationship existed between serum biochemistry and serostatus such that rabbits in poorer condition are more likely to be seropositive for MV and RHDV; and 3) to establish baseline values for biochemical parameters of rabbits with different serostatus in wild rabbit populations.

Materials and methods

Sampling

From autumn 2008 to spring 2010, we conducted seven live-trapping sessions in each enclosure. From a total 6605 rabbits captured, we selected 720 adult rabbits to take blood samples. Samples were analyzed to obtain anti-MV and anti-RHDV serum antibody concentrations (see further details on serology protocols in general materials and methods) and also to characterize biochemical parameters.

Biochemical analyses

We processed the serum samples using a COBAS INTEGRA 400 plus analyzer (Productos Roche España, Madrid, Spain).We determined the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BILI), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), urea (BUN), creatinine (CREA), albumin (ALB), and total proteins (TP). These biochemical parameters are indicators of organ function, protein metabolism, and nutritional status, which means they should be good proxies for physiological condition (Harder and Kirkpatrick, 1994; Stirrat, 2003). Since they were expressed in different units, they were transformed prior to analysis to enable comparisons.

Biochemical and immunological analyses were performed by the Physiological Ecology Laboratory of the Doñana Biological Station (Seville, Spain).

Data analysis

All statistical analyses were performed using R version 3.0.1 (R Core Development Team, 2013). Employing generalized linear mixed models (GLMM, glmer function, lme4 package) with a binomial distribution and a logit link function, we tested the relationships between the different biochemical parameters and serostatus for individuals in the three enclosures. To reduce heterogeneity, we limited our analyses to adults. Three sets of analyses were performed: 1) using rabbits seropositive for MV; 2) using rabbits seropositive for RHDV and 3) using rabbits seropositive for both MV and RHDV. To avoid possible confounding effects, in all the analyses, we considered that individuals were seronegative only if they had neither anti-MV nor anti-RHDV antibodies. Correlations among biochemical parameters were tested, and ALT, GGT, BILI, CREA, BUN, and TP were retained as predictor variables in the

subsequent analyses. Serostatus was the response variable. We also included sex and rabbit density as predictor variables in the models. Some individuals were sampled more than once by chance. To account for the increase in type I error (rejection of the null hypothesis when it is true) due to pseudoreplication (Hurlbert 1984), we included the following random variable: capture session nested within individual identity nested within enclosure number.

Prior to running the analyses, all the numeric predictor variables were scaled (except for "sex") using the scale function so that their relative importance could be compared. We selected the best-fit models via backward stepwise selection (anova function with maximum likelihood, Crawley 2012; $p < 0.05$ as the threshold value). Each of the final models contained only the significant predictors.

Results

Through the course of the study, we got samples from720 adult rabbits (274, 242, and 204 rabbits in E1, E2, and E3, respectively). A total of 346 samples were seropositive only for MV, 101 samples were seropositive only for RHDV, 200 samples were seropositive for both, MV and RHDV, and 245 samples were seronegative. Some individuals were sampled more than once and not always had the same antibody titre that is why the number of samples obtained does not math with the total number of individual animals handled.

None of the biochemical parameters analyzed were significantly associated with MV or RHDV serostatus. The only significant relationships we found were a positive association between rabbit density and MV seropositivity ($p < 0.001$; Table 1) and a negative association between GGT levels and seropositivity for both viruses ($p < 0.05$; Table 1).

Table 1. Results for the generalized mixed models for each dataset (i.e., MV: rabbits seropositive for myxoma virus; RHDV: rabbits seropositive for rabbit haemorrhagic disease virus; MV & RHDV: rabbits seropositive for both viruses). Coefficient estimates (β), estimated standard errors (SE), and p-values (p) are listed. indicates statistically significant value, P<0.005.*

	MV			RHDV			MV & RHDV		
	β	SE	p	β	SE	p	β	SE	p
ALT	0.2042	0.1241	0.09433	0.1940	0.1646	0.2486	0.2391	0.1362	0.08241
GGT	0.01504	0.11533	0.8961	-0.06207	0.17053	0.7131	-0.3031	0.1599	0.0491*
BILI	-0.07875	0.10509	0.453	-0.2079	0.1636	0.192	-0.1320	0.1427	0.3639
CREA	-0.04941	0.10756	0.6478	-0.03273	0.17178	0.8467	-0.1195	0.1651	0.4781
BUN	0.04409	0.10948	0.686	-0.01682	0.14124	0.9006	0.07518	0.11388	0.5182
ALB	-0.04380	0.11618	0.7065	0.02039	0.16918	0.8993	0.08296	0.14723	0.5847
density	0.3728	0.1112	0.000804*	-0.03413	0.17652	0.8461	0.01127	0.14411	0.9383
sex	0.2173	0.2155	0.3112	0.10556	0.29962	0.7248	0.3539	0.2554	0.1703

Each enclosure displayed different seroprevalence patterns (Figure 1). In E1, the percentage of rabbits seropositive for MV, RHDV, or both remained fairly constant over time. More specifically, MV seroprevalence was high for most of the trapping sessions. In contrast, RHDV seroprevalence was low; it peaked at 24.4% in session 3. In E2, the percentage of seronegative rabbits was generally higher than in E1 and E3, with values reaching a maximum of 63.8 and 69.4% in sessions 1 and 2. MV seroprevalence increased from session 3 to session 7, whereas the percentage of seronegative rabbits clearly declined. Remarkably, no rabbits were seropositive for RHDV in session 7. In E3, there was a higher percentage of individuals that were seropositive for both viruses, as compared to E1 and E2. It was also the enclosure with the lowest percentage of seronegative rabbits; this value climbed as high as 55.6% in session 4. Notably, there were no seronegative rabbits in sessions 1 and 7. Rabbits seropositive for MV and for RHDV were observed in every session, but their percentages were rather low. RHDV seroprevalence peaked in all three enclosures in session 3.

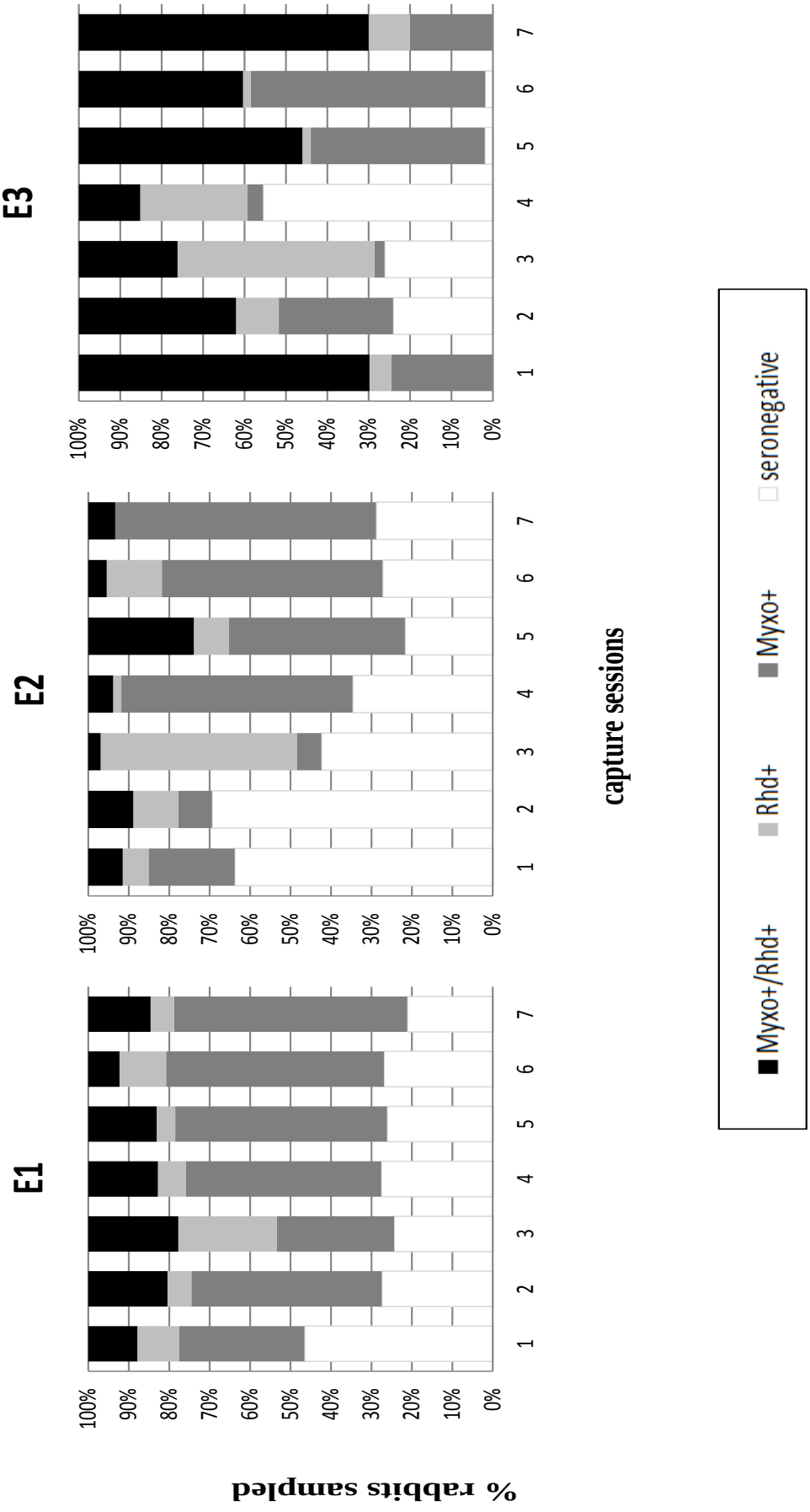


Figure 1. Variation in MV and RHDV seroprevalence in rabbit populations (E1, E2, and E3) over the two-year study period.

In general, the ranges of values observed for the biochemical parameters remained fairly consistent, although some noticeable changes in certain parameters occurred during certain capture sessions (Figure 2). In E1, most of the biochemical parameters had relatively constant values, but GGT and BILI fluctuated slightly. The pattern in E2 was more heterogeneous. Almost all the parameters varied somewhat, except for BUN, TP, ALB, and BILI. In the case of the transaminases—ALT, AST, and GGT—maximum values occurred in sessions 2, 5, and 7. CREA levels were fairly constant over time but hit a low in session 3, which coincided with the minimum values for the transaminases. Blood biochemistry patterns were most distinct in E3. As in E2, BUN, TP, ALB, and BILI varied little while the transaminases and CREA fluctuated dramatically. ALT and AST followed parallel patterns, both peaking in sessions 4, 6, and 7 and dropping to their minimum values in session 2. GGT presented an irregular pattern—levels were highest in session 4 and dipped down in sessions 3, 5, and 7. While CREA tended to remain constant, it dropped sharply after peaking in session 5.

Table 2 provides the means for the different biochemical parameters for the different enclosures and seropositivity classes; it also gives more detailed information related to the aforementioned patterns.

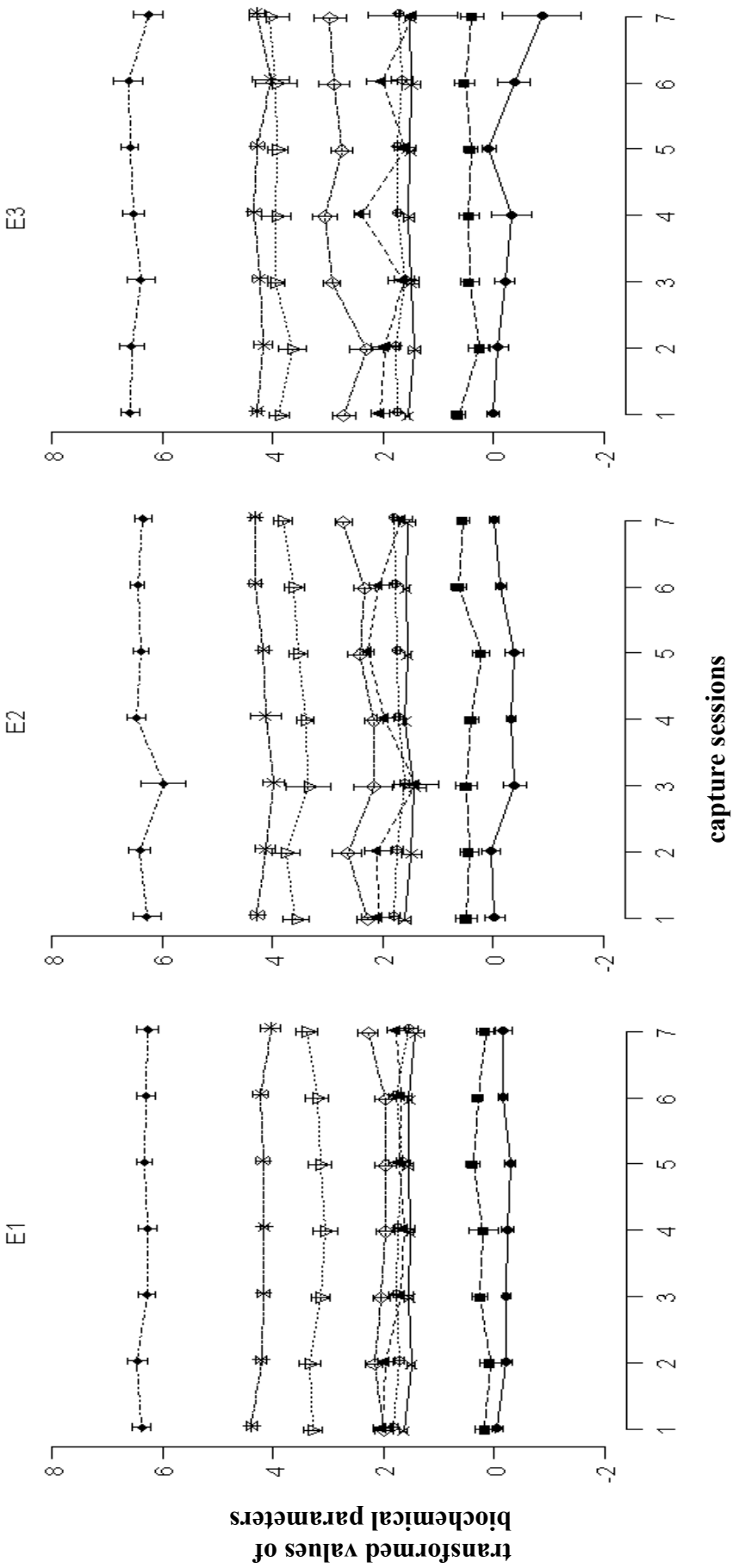


Figure 2. Transformed values (mean ± SE) of biochemical parameters for each capture session in the three study enclosures (E1, E2, and E3).Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BILI), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), urea (BUN), creatinine (CREA), albumin (ALB), and total proteins (TP).

Table 2. Blood biochemistry of wild European rabbits in the three study enclosures (E1, E2 and E3); rabbits are grouped by serostatus (seronegative, seropositive for myxoma virus [Myxo+], seropositive for rabbit haemorrhagic disease virus [Rhd+], and seropositive for both viruses [Myxo+/Rhd+]). Values correspond to the mean ± SE.

	E1				E2				E3			
Parameter(units)	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***
ALT (U/L)	9±0.7	8.5±0.9	9.6±0.5	10.1±0.9	12.6±0.8	14.7±1.8	13.8±1.0	15.1±2.0	19.6±2.1	20±2.0	21±1.4	20.3±1.2
AST (U/L)	32.2±3.2	31.4±3.2	31.9±1.9	38.1±5.0	44.8±3.0	46.4±6.3	48.3±4.1	39.8±3.9	66±9.1	56.8±6.1	60.8±4.0	65.3±5.1
GGT(U/L)	6.9±0.6	7±0.7	7.4±0.4	7.1±0.6	9.2±0.6	8±1.1	8.4±0.5	8±0.8	9.2±0.9	9.3±0.9	10.3±1.3	7.4±0.4
BILI (µmol/L)U/L)	1.4±0.1	1.2±0.1	1.4±0.1	1.3±0.1	1.7±0.1	1.6±0.1	1.7±0.1	1.5±0.2	1.7±0.2	1.7±0.1	1.8±0.1	1.7±0.1
CREA (mg/dl)	0.8±0.04	0.8±0.07	0.8±0.02	0.8±0.05	0.9±0.04	0.8±0.07	0.8±0.05	0.9±0.1	1±0.2	0.9±0.1	1±0.1	0.9±0.05
BUN(mg/dl)	73.7±4.8	76.2±7.9	74±2.2	71.9±3.4	70.4±2.6	66±4.9	77.7±2.3	73.2±5.7	76.5±5.1	77.7±3.3	72.4±3.3	79.8±3.4
LDH(U/L)	643.1±43.5	647.7±34.2	697.8±44.0	697.4±64.6	670.5±37.9	582.9±52.4	703±36.9	740±91.8	890±98.0	743.2±95.7	918.9±73.2	862.4±63.5
ALB(g/dl)	4.8±0.1	5±0.2	4.7±0.1	4.7±0.1	4.9±0.1	4.5±0.2	4.9±0.1	5±0.1	4.5±0.2	4.7±0.1	4.7±0.1	4.6±0.1
TP (g/dl)	5.7±0.2	6.1±0.4	5.7±0.1	6±0.4	5.8±0.1	5.4±0.2	5.8±0.1	5.7±0.3	5.4±0.2	5.7±0.1	5.8±0.1	5.8±0.1

Discussion

To our knowledge, this is the first study conducted in the field to address the relationship between MV and RHDV seropositivity and the physiological status of wild European rabbits using large numbers of animals and in the context of a long-term monitoring program.

In light of the results, we found limited evidence for an association between blood biochemistry and serostatus in wild European rabbit populations. The only significant relationship we observed was that rabbits seropositive for both MV and RHDV had lower GGT concentrations (Table 1). However, the lack of significant findings might be due to spurious results generated by data heterogeneity and the presence of confounding variables.

One major methodological handicap is the scarcity of data on wild rabbit populations. Most studies dealing with myxomatosis and RHD have focused on disease pathology and epidemiology in domestic rabbits. Consequently, most of the information currently available has been obtained using rabbits reared under laboratory conditions (Calvete et al., 2002; Calvete et al., 2005; Cabezas et al., 2006; Kerr, 2012). However, physiological data for domestic rabbits is not directly comparable to that for wild rabbits since major differences exist in genetics, environmental contexts, breeding conditions, individual responsiveness, and even laboratory processes and techniques. In addition, laboratory rabbits usually develop physiological problems and specific pathologies as a result of living in captivity. These limitations aside, our results suggest that myxomatosis and RHD have declined in severity because they have become endemic in the Iberian Peninsula (Ross et al., 1986; Ross et al., 1989; Marchandeau et al., 1999; Calvete et al., 2002; Marchandeau et al., 2014). Endemic diseases have strong initial effects and cause high mortality rates in afflicted populations.

However, the individuals that survive experience constant reinfections over time, ultimately leading to high immunity levels within populations. As a result, individuals become partly protected and most show mild clinical symptoms throughout the year. The pathogen can then be said to be in permanent circulation and to have become enzootic (Calvete et al., 2002; Cooke, 2002; Fouchet et al., 2008). This state of affairs is consistent with our results (Figure 1). Although the three enclosures exhibited some distinct differences, in general, there were always some individuals seropositive for MV, RHDV, or both throughout the study period. This finding suggests that the two viruses are now endemic in the study populations. It is also worth noting the fluctuating percentage of seronegative rabbits seen in E3: there were no seronegatives at the beginning or at the end of the study period. In E2, no RHDV-seropositive rabbits were found in the last capture session. Individuals with severe RHDV infections might have died, leaving no seropositives in the population; consequently, new outbreaks may result in high mortality rates. This pattern might be linked to the severity of RHD and its relatively more recent arrival, as compared to myxomatosis.

When we looked at the results for rabbit biochemistry and serostatus in tandem for the different enclosures, we observed that both were highly homogenous in E1. In E2, transaminases and CREA peaked in session 2, which was when the percentage of seronegative rabbits was the highest. The number of seronegative rabbits declined over subsequent sessions, while rabbits seropositive for MV, for RHDV, and for both became more abundant. One possible explanation is that E2 rabbits were exposed to the viruses around the time of session 2 (there were a number of outbreaks that season, as described in the literature [i.e., Calvete et al., 2002]), which is suggested by the session 2 peak in transaminases. In sessions 3 and 4, the number of seropositive rabbits increased and both the transaminases and CREA dropped to their minimum values.

This result lends support to the idea that rabbits that have been exposed to the viruses, and that consequently develop immunity, are likely to return to basal biochemical parameter values.

In E3, transaminases peaked in session 4, which is also when the number of seronegative rabbits was highest. In the subsequent capture sessions (sessions 5 and 6), the percentage of seronegative individuals declined sharply while the number of individuals seropositive for MV, RHDV, or both climbed. This pattern probably resulted from a high incidence of the diseases in session 4 and earlier. The population's exposure to the viruses can be seen in the increase in transaminases in session 4, which is when they reached maximum levels. In sessions 5, 6, and 7, after rabbits had become seropositive, the transaminases were close to their minimum levels, suggesting that immune (seropositive) rabbits tended to return to basal parameter levels.

As in E2, in E3 there were large numbers of seronegative rabbits in sessions 2 and 3, just before transaminases peaked in session 4, which likely signaled the beginning of an endemic disease cycle.

Of the biochemical parameters studied, the transaminases (ALT, AST, and GGT) were clearly the most variable for all three enclosures. This pattern may reflect the impaired hepatic function seen in rabbits infected with MV and/or RHDV. In addition to the shortcomings mentioned above, the lack of significant findings puts into question the utility of biochemical parameters in assessing the physiological condition of European rabbits. As is clear from the literature, serum biochemistry might be influenced by a variety of factors, including rabbit handling and sampling procedures, fieldwork conditions, and animal nutritional and health status at the time of sampling (Calvete et al., 2005; Cabezas et al., 2006). Furthermore, there is individual-level variation in immune and physiological responses as a result of trade-offs between environmental conditions and life-history traits (e.g., developmental, physiological,

genetic, and immunological traits) (Ardia et al., 2011). Therefore, alternative indicators such as concentrations of specific immunoglobulins (e.g., IgM or IgG) or cellular oxidative stress markers could provide more complete and precise information. As discussed above, confounding variables that were not accounted for in our analyses could be skewing our results. Such variables could include the following: (1) rabbit age; (2) outbreak timing; (3) the ELISA seropositivity thresholds; (4) the response speed of biochemical parameters; and (5) the lack of reference values for wild rabbits.

One major factor could be rabbit age. In this study, we estimated age based on mass. Although this approach can separate adult rabbits from non-adult rabbits, it cannot reveal a rabbit's precise age. Knowing a rabbit's age could be important because as rabbits get older, their probability of being infected by a wide variety of potentially serious pathogens like MV or RHDV increases, as do antibody levels (Marchandeau et al., 1995; Parkes et al., 2002; Parkes et al., 2008). Furthermore, a rabbit's innate responsiveness changes over its lifetime, which means that individuals of different ages will have different biochemical profiles and immunological experience.

Outbreak timing is also important but difficult to characterize. Myxomatosis and RHD outbreaks show some seasonal and geographic variation (Mutze et al., 2008; Mutze et al., 2010; Abrantes et al., 2012). More specifically, the occurrence of epizootics might vary across years and even among populations (i.e., enclosures) as a result of delayed breeding and variable climatic conditions, which can affect the abundance and activity of the pathogens' vectors. Determining the moment of infection is nearly impossible, so outbreak timing is only approximate.

As mentioned above, wild rabbits are naturally exposed to a wide variety of pathogens, whereas laboratory rabbits are artificially infected with a smaller selection of

them. The ELISA techniques that we used to determine MV and RHDV seropositivity were developed using European rabbits kept under laboratory conditions. It may be that applying such seropositivity thresholds to wild rabbits could yield false positives and cross-reactions since laboratory rabbits are exposed to fewer pathogen species and thus have lower threshold antibody concentrations than wild rabbits (Kerr, 1997).

Finally, the response speed of biochemical parameters must be accounted for. Serum biochemistry changes are relatively transient, as demonstrated by several studies in which rabbits were artificially infected with pathogens (Ferreira et al., 2004). Rabbits show an initial physiological response to infection, but if they do not die, any changed biochemical parameters revert to their basal values. Nevertheless, such shifts are likely to go undetected in the wild.

In conclusion, it will be important to carry out further research that explores straightforward, reliable indices that can be used to assess the physiological condition of individuals in target wildlife populations. Selecting the right methods and biochemical parameters is essential if we wish to more rapidly detect and control diseases in wild species, which would help improve management and conservation programs.

CHAPTER 4

Oxidative stress in wild European rabbits naturally infected with myxoma virus and rabbit haemorrhagic disease virus

European Journal of Wildlife Research (Under Review)

Abstract

The European rabbit (*Oryctolagus cuniculus*) is one of the most important vertebrate species in the Mediterranean Basin ecosystem. Over the last 60 years, the arrival of two viral diseases, myxomatosis and rabbit haemorrhagic disease, have led to dramatic declines in wild rabbit populations across the Iberian Peninsula. These diseases are currently endemic. Periodic outbreaks occur and have significant impacts on wild populations. Both infection types have diverse physiological effects on their hosts that are rooted in aerobic metabolic processes. To fight off these viruses, rabbits activate their immune systems. However, the production of immune defences generates reactive oxygen species that may consequently damage host tissues. Hypothesising that immune responses increase oxidative stress, we examined whether wild rabbits naturally infected with myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) had high oxidative stress. Using blood samples, we measured anti-MV and anti-RHDV antibody concentrations and different oxidative stress markers (i.e., glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, and malondialdehyde).Our results show that rabbits that were seropositive for both MV and RHDV had high concentrations of malondialdehyde. Age and body condition were also positively related to dual seropositivity. No significant relationships were observed between serostatus and the concentrations of the other oxidative stress markers. Although we expected infection with MV and RHDV to be correlated with oxidative stress, the influence of external sources of oxidative stress (e.g., climatic conditions) likely made it more difficult to detect such relationships in wild rabbits.

Introduction

The wild European rabbit (*Oryctolagus cuniculus*) is an important keystone species in the Mediterranean Basin ecosystem. Over the past several decades, its populations have undergone a sharp decline in the Iberian Peninsula, largely due to the impact of two major viral diseases: myxomatosis and rabbit haemorrhagic disease (RHD) (Calvete et al., 2002; García-Bocanegra et al., 2010). Myxomatosis and RHD are currently endemic; significant annual mortality is caused by myxomatosis outbreaks in summer and autumn and RHD outbreaks in spring and winter (Calvete et al., 2002; Villafuerte et al., 2017). Both myxoma virus (MV) and RHD virus (RHDV) can induce significant physiological stress because host metabolic rate climbs during infection (Tuñon et al., 2003; Sanchez-Campos et al., 2004; Lastra, 2009; Costantini, 2014).

In general, immune responses draw upon metabolic processes, increasing immunity and disease resistance at an energetic cost (Lochmiller and Deerenberg, 2000). There are also biological fitness costs (Costantini, 2008). To fight these pathogens, rabbits first activate an innate inflammatory immune response. Inflammation is a non-specific process during which fluids, compounds, and immune cells (e.g., phagocytes) are disseminated through the bloodstream to damaged or infected tissues (Sorci and Faivre, 2009). Phagocytes release reactive oxygen species (ROS), particularly during neutrophil bursts, to neutralise pathogens (Klebanoff and Clark, 1978; Swindle and Metcalfe 2007). ROS also play an important role in cell signalling and regulation (Thannickal and Fanburg, 2000; Dröge, 2002; Costantini, 2014). However, ROS have dose-dependent effects (Gechev et al., 2006; Quan et al., 2008). At low levels, they serve as important molecular messengers in biological processes (Apel and Hirt, 2004; Foyer and Noctor, 2005; Gechev et al., 2006); at high levels, though,

they can induce DNA, lipid, and protein damage (Harman, 1956; Beckman and Ames, 1998; Dowling and Simmons, 2009; Selman et al., 2012). Therefore, although ROS can act as highly effective antimicrobial agents, they can also potentially damage host tissues and cells. Such negative side effects can be counteracted by a complex antioxidant (AOX) system that consists of a wide range of endogenous and exogenous compounds (Halliwell and Gutteridge, 1999; van de Crommenacker et al., 2010), although any imbalance between ROS and AOXs in favour of the former gives rise to oxidative stress (OS) (Sies,1991; Halliwell and Gutteridge, 2007). OS causes gradual deterioration of organismal function and cell senescence over time and is thus believed to be an important modulator of life-history trade-offs in vertebrates (Costantini, 2008; Nussey et al., 2009; Costantini, 2014).

Therefore, an organism's OS level plays an important role in reproductive performance and aging, which means it is also significantly linked to body condition and biological fitness (Beckman and Ames, 1998; Hulbert et al., 2007). Evaluating OS levels is therefore an important part of determining an individual's health status. Since both plasma and serum markers can reveal exposure to stress, biochemical assays that characterise blood oxidative profiles could be used to determine the extent of OS in wild vertebrates (Costantini, 2008). According to recent findings, at least one marker of AOX activity and one marker of oxidative damage should be measured to adequately quantify overall OS (Prior and Cao, 1999; Clarkson and Thompson, 2000; Cohen and McGraw, 2009; Costantini and Verhulst, 2009; Selman et al., 2012; Christensen et al., 2015).

Laboratory and field studies have revealed that mounting an immune response can increase oxidative damage and decrease AOX activity (Tanchev et al., 2003; Costantini and Moller, 2009; Marri and Richner, 2015; von Schantz et al., 2016).

However, despite evidence suggesting that infection risk, OS, and host immune capacity are related, little is known about the temporal consistency of relationships among viral infections, OS markers, and immune function in wild animals.

In this study, we tested the hypothesis that immune responses are associated with oxidative stress using three wild populations of the European rabbit. We measured anti-MV and anti-RHDV antibody concentrations as well as markers of AOX activity and oxidative damage in serum samples collected over six sampling periods. The goal of our study was to improve our understanding of immune response dynamics and changes in OS and, more specifically, to determine how animals could physiologically cope with oxidative damage resulting from a natural immune challenge. We wished to clarify any potential harmful effects on body condition and biological fitness that could threaten the viability of natural populations.

Materials and methods

Sampling

Animals were captured seasonally in the three enclosures of study: six live-trapping sessions were conducted from autumn 2008 to spring 2010. From a total 6605 rabbits captured, we selected 669 adult rabbits to take blood samples. Samples were analyzed to obtain anti-MV and anti-RHDV serum antibody concentrations (see further details on serology protocols in general materials and methods) and also to assay OS markers.

Oxidative stress analyses

Using the serum samples, we assayed five different OS markers: four AOX enzymes—glutathione peroxidase (GPX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT)—and one compound that signals oxidative damage—malondialdehyde (MDA; a product of lipid peroxidation).

GPX (U/mg of protein) is an enzyme that catalyses the reduction of hydrogen peroxide (H_2O_2) and a wide variety of organic peroxides (R-OOH) into their corresponding stable alcohols (R-OH) and water using cellular glutathione as the reducing reagent. Its concentration was determined by estimating NADPH oxidation as per Carmagnol et al. (1983). GR (mU/mg of protein) is a flavoprotein that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) into glutathione (GSH). Its concentration was determined as per Cribb et al. (1989). SOD (U/mg of protein) is a metalloenzyme that catalyses the dismutation of the superoxide anion into either molecular oxygen or hydrogen peroxide; it is thus a crucial part of the cellular antioxidant defense mechanism. Its concentration was determined as per McCord and Fridovich (1969). CAT (U/mg of protein) is an enzyme that catalyses the decomposition of hydrogen peroxide into water and oxygen and is a very important enzyme in oxidative metabolism. Its concentration was determined as per Cohen et al. (1969). Finally, MDA (nmol/ml) is a low-molecular-weight molecule that is the end-product of the peroxidative decomposition of unsaturated lipids. Its concentration was determined using the Buege and Aust method (1978).

All analyses were carried out in the Laboratory of Ecophysiology at the Doñana Biological Station (CSIC, Seville, Spain).

Body condition

To estimate rabbit body condition (BC), we calculated a scaled mass index (Peig and Green, 2009). This index standardises body mass based on a chosen metric of body length (tarsus length in our case) and is designed to account for allometric changes in scaling that are observed in many species (Gibbs and Chiucchi, 2012). BC was used in combination with serostatus to assess rabbit health costs.

To calculate BC, mass (in grams) and tarsus length (TL, in mm) were log-transformed, and Model II Regression (lmodel2 package in R v. 3.3.2; Legendre, 2008) was used to calculate the slope (b_{SMA}) of the best-fit line using major axis regression. L_o was calculated; it was the mean value of TL based on the entire dataset (mean TL females=71.7 mm; mean TL males=72.8 mm). We calculated separate scaled mass indices for female and male rabbits because, as mentioned above, rabbit age was based on mass and patterns differed between the sexes.

Furthermore, gravid females were excluded because they were expected to be heavier relative to their length, which could have biased the calculations.

Data analysis

We performed generalised linear models (GLMs, stats package in R v. 3.3.2) with binomial error distributions and logit link functions. Serostatus was the response variable (rabbits seropositive for MV, rabbits seropositive for RHDV and rabbits seropositive for both MV and RHDV); the explanatory variables were the serum concentrations of the OS markers (GPX, GR, SOD, CAT, and MDA). We also included age, sex, BC, and enclosure density as predictors. To minimize data heterogeneity, the

numeric explanatory variables were log-transformed before the models were run. Correlations among variables were determined to select the explanatory variables included in the final model.

All statistical analyses were performed using R software (v. 3.3.2; R Core Team, 2016).

Results

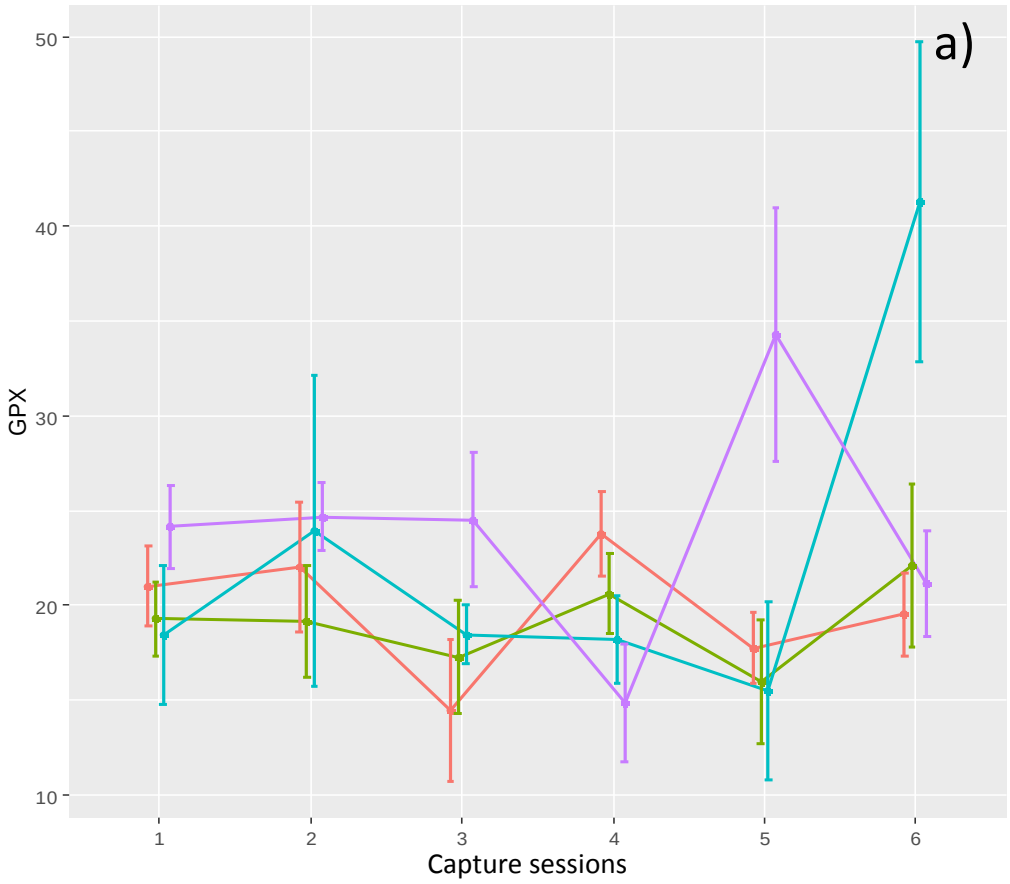
We obtained a total of 669 blood samples from 589 adult and 80 juvenile rabbits (264, 203, and 202 rabbits in enclosures E1, E2, and E3, respectively). A total of 221 samples were seropositive for MV alone, 86 samples were seropositive for RHDV alone, 133 samples were seropositive for both MV and RHDV (dual seropositivity), and 229 samples were seronegative.

None of the OS marker concentrations were significantly associated with MV or RHDV serostatus alone. Indeed, we detected one significant positive relationship: between MDA concentrations and dual seropositivity (MV/RHDV; $p < 0.01$; Table 1). Both age ($p < 0.001$; Table 1) and BC ($p < 0.01$; Table 1) were positively associated with dual seropositivity. Age ($p < 0.001$; Table 1), BC ($p < 0.001$; Table 1), and rabbit density ($p < 0.01$; Table 1) showed a positive relationship with RHDV seropositivity.

Table 1. Results of the different generalised models in which serostatus was the response variable (i.e., MV: myxoma virus serostatus; RHDV: rabbit haemorrhagic disease virus serostatus; MV/RHDV: dual serostatus). Coefficient estimates (β), estimated standard errors (SE), and p-values (p) for each explanatory variable (i.e. BC, Body condition Index; GPX, glutathione peroxidase; GR, glutathione reductase; SOD, superoxide dismutase; CAT, catalase; MDA, Malondialdehyde) are provided.

	MV			RHDV			MV & RHDV		
	β	SE	p	β	SE	p	β	SE	p
BC	-0.31139	0.45666	0.4953	1.48716	0.51883	0.00415 **	1.30549	0.56197	0.020176 *
GPX	-0.17926	0.13615	0.1880	-0.22912	0.16447	0.16358	-0.30821	0.16804	0.066640 .
GR	-0.23550	0.36100	0.5142	-0.88604	0.49678	0.07449 .	-0.81682	0.44832	0.068462 .
SOD	0.38006	0.38473	0.3232	0.26027	0.49502	0.59905	0.27198	0.44435	0.540488
CAT	-0.28902	0.33474	0.3879	-0.02029	0.47941	0.96625	-0.04903	0.39931	0.902275
MDA	0.35133	0.51307	0.4935	0.55225	0.65826	0.40149	1.31430	0.60213	0.029053 *
age	3.48721	0.74080	2.51e-06 ***	0.13719	0.35693	0.70072	3.87446	1.03321	0.000177 ***
sex	0.08235	0.20971	0.6945	-0.10664	0.26625	0.68878	-0.27910	0.24339	0.251505
dens	0.27995	0.12526	0.0254 *	-0.09578	0.20385	0.63845	-0.31416	0.16189	0.052311 .

(0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1).


a)
GPX
50
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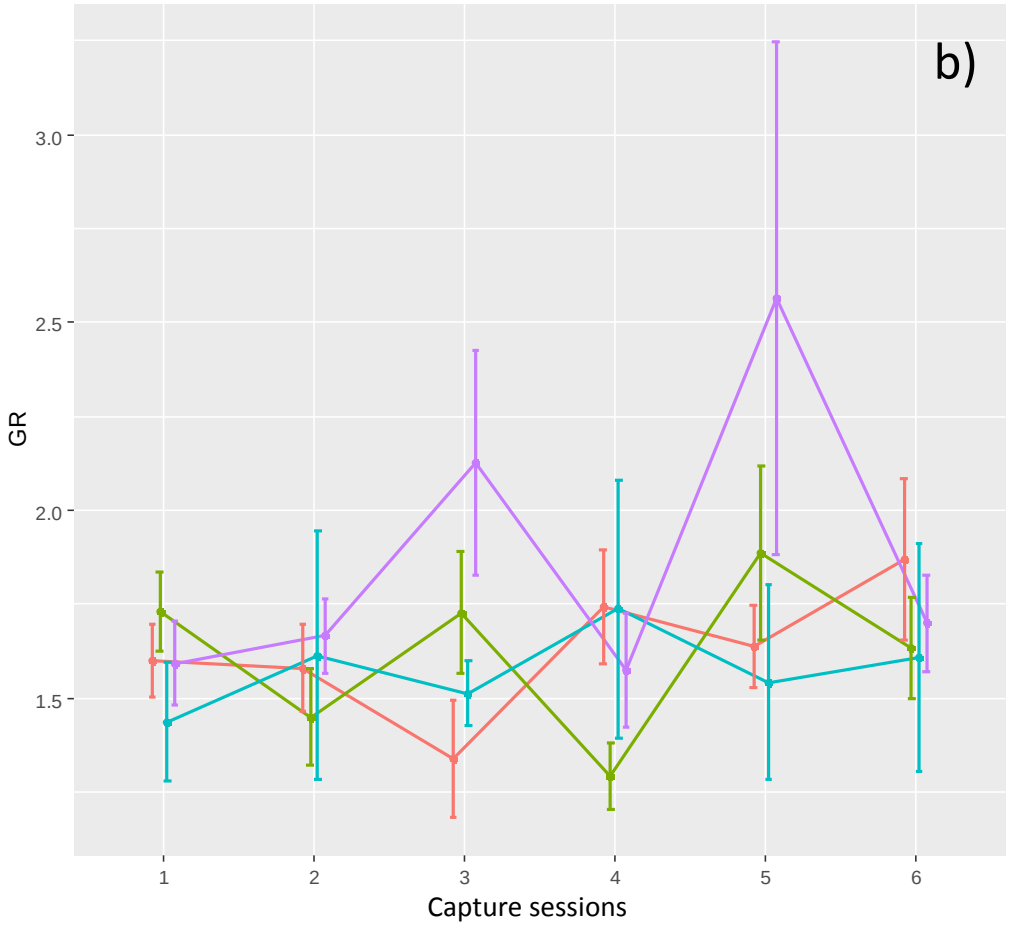
Capture sessions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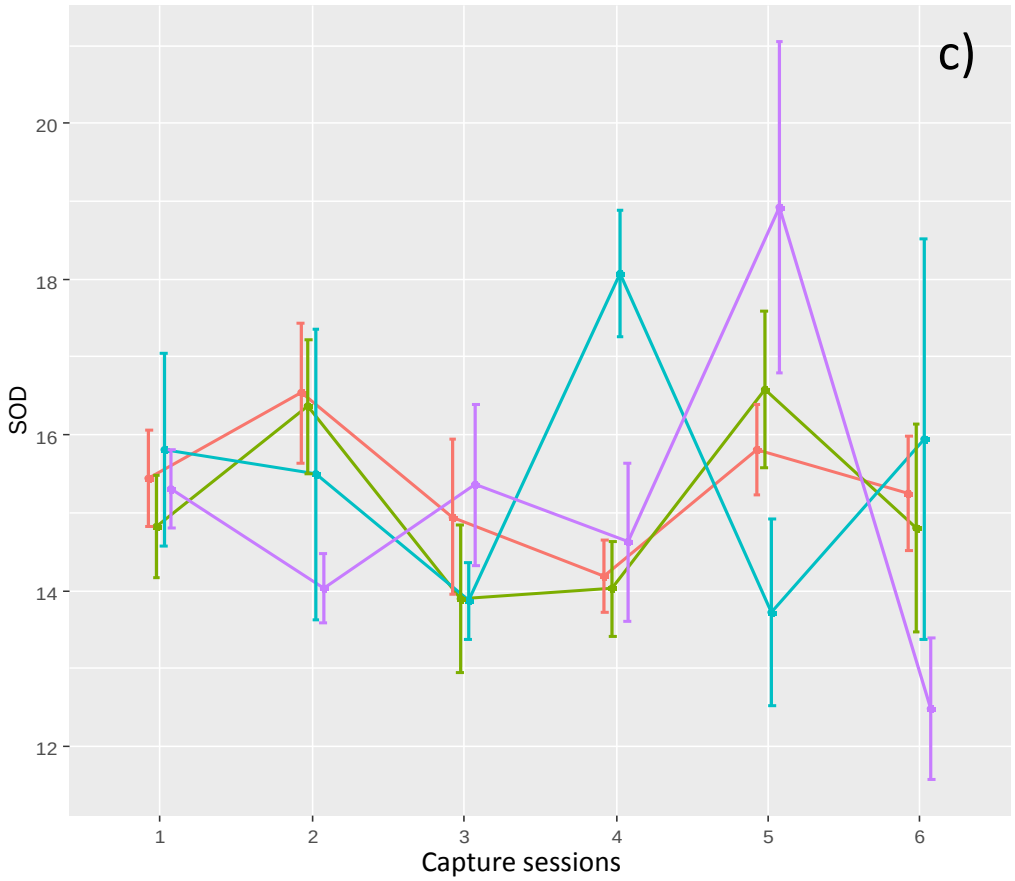
c)
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12
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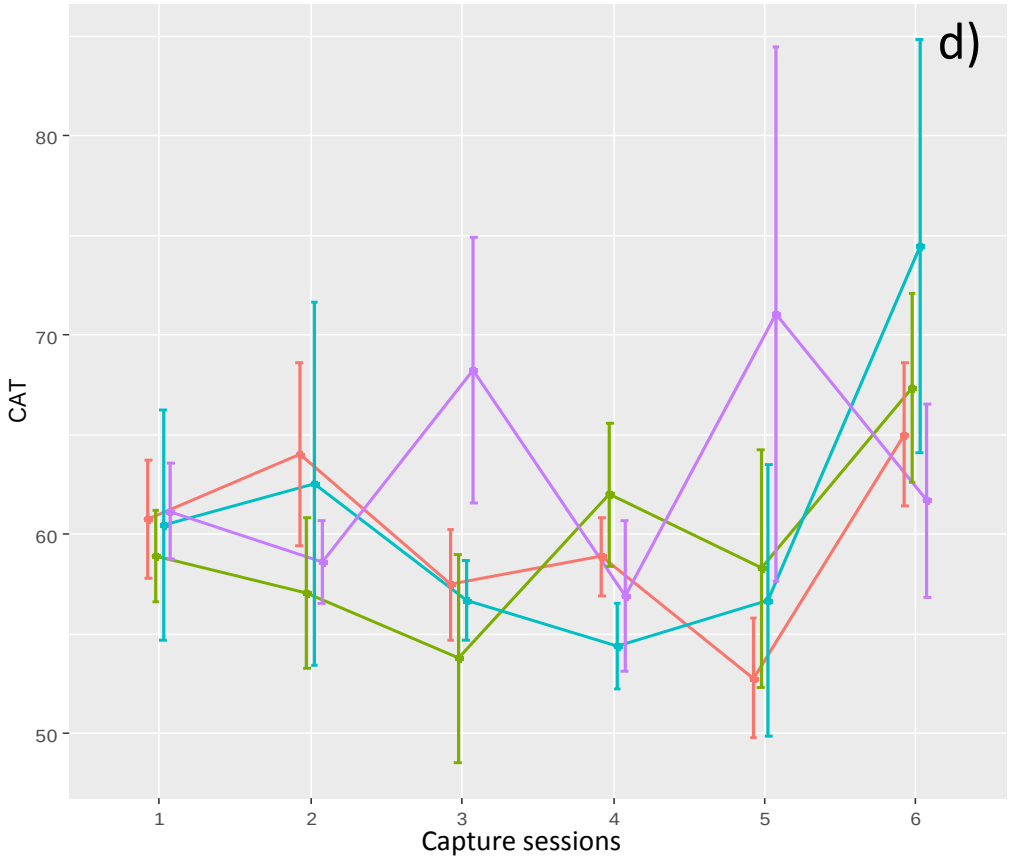
d)
CAT
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Capture sessions

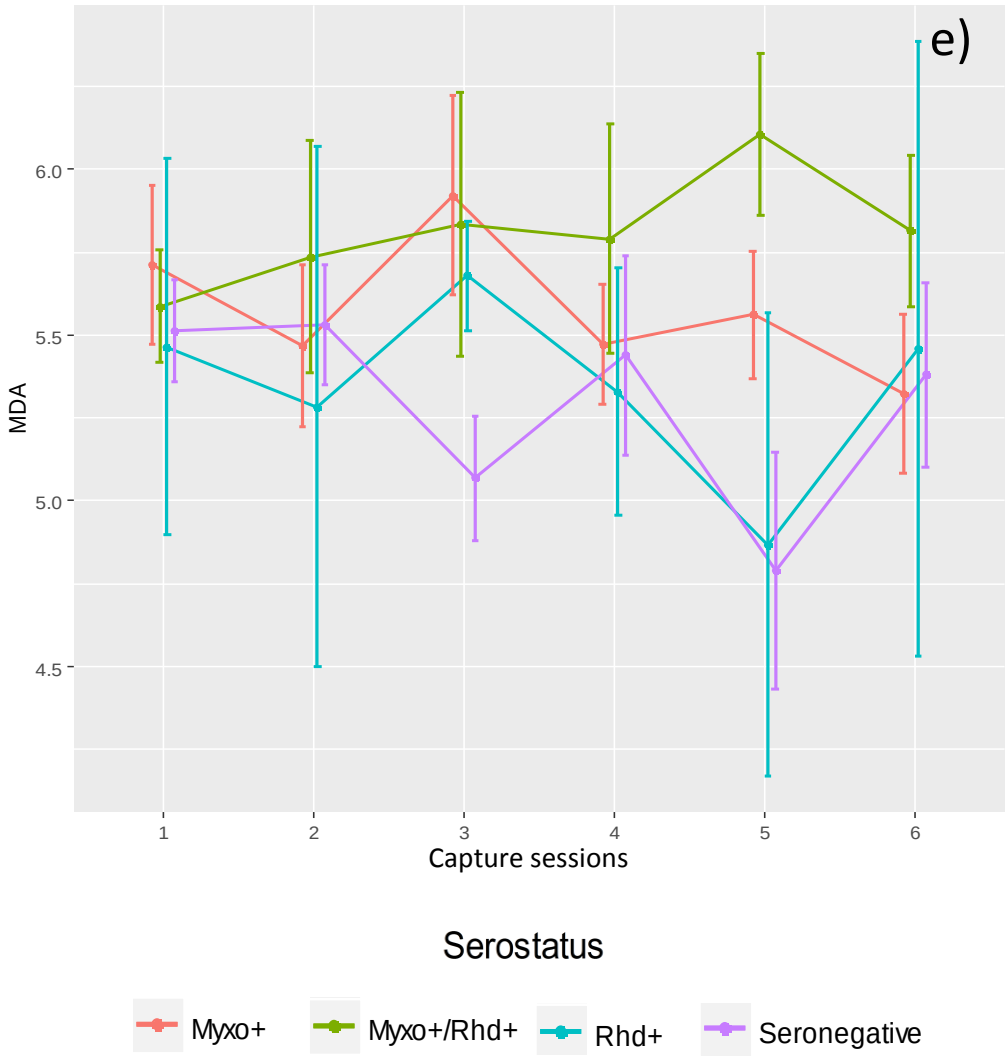


Figure 1. Average concentrations of OS markers (mean ± SE) across capture sessions based on rabbit serostatus categories: seronegative, seropositive for myxoma virus [Myxo+], seropositive for rabbit haemorrhagic disease virus [Rhd+], and seropositive for both viruses [Myxo+/Rhd+]. The marker abbreviations are as follows: a) glutathione peroxidase (GPX), b) glutathione reductase (GR), c) catalase (CAT), d) superoxide dismutase (SOD), and e) malondialdehyde (MDA).

Concentrations of OS markers were quite consistent over time. Overall, markers of AOX activity (GPX, GR, SOD, and CAT) were present in low concentrations in individuals that were seropositive for MV alone, RHDV alone, and both MV and RHDV (Fig. 1). In contrast, MDA concentrations were high in seropositive individuals (MV, RHDV, and MV/RHDV) and low in seronegative individuals (Fig. 1).

Table 2 provides the descriptive statistics for the OS marker concentrations for each enclosure and each serostatus group.

Table 2. Concentrations of oxidative stress (OS) markers in wild European rabbits in three study enclosures (E1, E2, and E3). Results are grouped by rabbit serostatus (seronegative, seropositive for myxoma virus [Myxo+], seropositive for rabbit haemorrhagic disease virus [Rhd+], and seropositive for both viruses [Myxo+/Rhd+]). The values are the mean ± SE.

	E1				E2				E3			
Parameter(units)	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***	***seronegative***	***Myxo+***	***Rhd+***	***Myxo+/Rhd+***
GPX (mU/mg prot)	24.4±2.2	18.3±2.4	20.8±1.5	18.9±2.2	22.9±1.8	20.2±2.4	21.5±1.7	21.3±2.9	26.0±2.5	19.7±2.4	19.8±2.2	19.1±1.4
GR (mU/mg prot)	1.8±0.2	1.5±0.1	1.8±0.1	1.6±0.1	1.9±0.2	1.6±0.1	1.5±0.1	1.5±0.1	1.7±0.1	1.5±0.1	1.7±0.2	1.6±0.1
SOD (U/mg prot)	15.8±0.6	16.4±0.6	15.8±0.4	15.4±0.5	15.1±0.6	14.1±0.8	14.8±0.5	16.4±1.0	13.0±0.5	13.1±0.5	14.7±0.5	14.2±0.5
CAT (U/mg prot)	64.5±3.5	59.6±3.2	61.6±1.7	60.2±2.5	62.3±3.3	56.9±2.8	62.0±2.9	63.9±4.6	57.8±2.3	57.5±2.8	51.9±2.5	58.1±2.1
MDA (nmol/ml)	5.5±0.1	5.6±0.3	5.7±0.1	5.8±0.2	5.4±0.1	5.3±0.2	5.3±0.2	5.9±0.5	5.1±0.2	5.6±0.3	5.3±0.2	5.7±0.2

Discussion

To our knowledge, this study is the first to look at the relationships between MV and RHDV seropositivity and OS in wild European rabbit populations.

We found no association between the concentrations of AOX markers and serostatus. One possible explanation is that OS may be relatively minor in wild rabbits infected with MV and/or RHDV because other environmental factors (e.g., climatic conditions, food availability, and parasite infections) have a much greater impact on an animal's oxidative status (Gassó et al., 2016). However, rabbits seropositive for both MV and RHDV did have high concentrations of MDA in their sera (Table 1; Fig. 1). This result could lend support to the idea that rabbits infected with MV and/or RHDV show augmented ROS and oxidative damage may arise as a consequence. Other factors such as BC, age, and enclosure density were significant (Table 1). Interestingly, age was positively associated with both MV seropositivity and dual seropositivity, but not with RHDV seropositivity. However, this latter result makes sense given RHDV's lethality. Santoro et al. (2014) found that rabbits seropositive for RHDV had low survival rates; juveniles were more susceptible than adults because of their immature immune systems. Consequently, high mortality in young age classes would mean that a high percentage of individuals failed to reach adulthood. BC was positively correlated with RHDV seropositivity and dual seropositivity but not with MV seropositivity. It is worth nothing that rabbits seropositive for MV have higher survival rates than rabbits that are seronegative (Santoro et al., 2014). Nevertheless, the strong immunosuppressive effects

of myxomatosis can favour opportunistic infections (Cattadori et al., 2008), and hence BC may be of minimal importance.

We would like to acknowledge some of the limitations of the current study. Assessments of OS levels can be highly variable due to taxonomic differences, different environmental conditions, or the short-term nature of many sampling efforts (e.g., because of logistical constraints). Consequently, they may not provide the best evidence for addressing the questions we have raised (Costantini et al., 2009; Norte et al., 2009; van de Crommenacker et al.,2011; Raja-aho et al., 2012; Rubolini et al., 2012; Pap et al., 2014).

We thus wish to emphasise the need for experimental studies, which could elucidate mechanisms underlying OS and facilitate the interpretation of field-study results (Costantini, 2008; Monaghan et al., 2009; van de Crommenacker, 2010). That said, this study is important because it examines the ability of OS to serve as a proxy for the body condition costs that wild rabbits pay when infected with these viral diseases. It is crucial to conduct further research to disentangle the consequences of oxidative damage on the health and biological fitness of wild European rabbits to ensure the viability of natural populations of this keystone species.

In conclusion, although AOX markers appeared to remain unaffected, we observed an increase in oxidative damage to lipids in rabbits seropositive for both MV and RHDV. This finding likely suggests that ROS levels were increased in naturally infected rabbits. That said, within wild populations, other factors may have a major impact on OS levels, potentially masking the contribution of viral infections.

At present, there is increasing interest in using OS markers to assess animal health status in ecological studies of wild populations. However, to date, they have

yielded mixed evidence, perhaps because of methodological variation, taxonomic differences, variable environmental conditions, or short sampling periods. Further research is needed to better understand the influence of pathogens on OS levels in wild animals and to unravel the consequences for individual biological fitness and population viability.

GENERAL DISCUSSION

As mentioned at the beginning of this thesis, emerging infectious diseases represent one important issue that wild species have to cope with. However, disease is a really complex process that rarely is associated to one single pathogen. Indeed, free-living wild rabbits must face a diverse array of factors as a result of dynamic interactions among many species of pathogens, parasites (i.e: biotic factors) and wide variety of environmental features (i.e: abiotic factors) (Wobeser, 2007). Such complex network determines the severity of disease symptoms, the epidemiology of the pathogen species and rabbit fitness within wild populations; thence it plays a pivotal role in the regulation of population dynamics (Pedersen and Fenton, 2007).

Essentially, it is important to recall the major impact that viral diseases (i.e: myxomatosis and RHD) and parasitosis (i.e: coccidiosis and helminthiasis) have on wild rabbit populations and consequently on the endangered predators that prey upon them. Even though the former diseases have become endemic, periodic outbreaks still occur and cause significant mortality rates (21-23% annual mortality rates, Calvete et al., 2002). Unfortunately, nowadays long-term epidemiological studies are not very numerous and often focus on descriptive aspects only, neglecting the interaction among different pathogens.

In this regard, the present thesis provides substantial progress in understanding mechanisms that underlie disease process and their effects on the survival of wild rabbit populations. The "tandem" composed by myxoma virus (MV) and rabbit haemorrhagic disease virus (RHDV) — causative agents of the principal viral diseases in the wild European rabbit — as well as their interplay with other parasites (i.e: coccidia and nematodes) is of special interest to study rabbit populations' trends. Here we gain further knowledge on how and to what extent these interactions affect rabbits' survival

patterns by assessing rabbit physiology through long-term monitoring of biochemical and oxidative stress parameters.

Rabbit survival, as observed in the **chapter 1**, is not absolutely determined by seropositivity. Despite protection conferred by antibodies, negative side effects (i.e: immunosuppression) have been described in rabbits infected with MV (Jeklova et al., 2008). Actually, Boag et al. (2013) reported that MV could compromise rabbit immunity to coccidia and nematodes, causing an increase in these parasites' loads. Immune response against macro-parasites (i.e: nematodes) normally elicits Th2 response, while immunity to micro-parasites (i.: MV, RHDV and coccidia) usually involves a Th1 response (Graham et al., 2003). Nevertheless, it has been suggested that in the MV-coccidian/nematode coinfection model the Th1/Th2 system is not completely polarized (Cattadori et al., 2007), observing MV infected-rabbits with high nematode loads. Contrary to results of Boag et al. (2013) and Marques-Silva et al. (2015), that supported our baseline hypothesis, we found a negative relationship between MV seroprevalence and coccidian load **(chapter 2).** The reason of such discrepancies could be in line with our data that came from adult rabbits exclusively constrained to restocking enclosures, whereas the preceding studies dealt with free-ranging rabbits of all ages (Boag et al., 2013). Also ecological and environmental conditions were quite different since the study areas that those authors selected were characterized by distinct climate conditions to the Mediterranean climate herein.

Characteristically, oocyst counts in wild rabbit populations show considerable variability through months and years (Stodart, 1968a, b; Hobbs et al., 1999b; Foronda et al., 2005); therefore, the coccidian loads we obtained in this thesis are likely to be distinct from the ones other authors reported as a consequence of natural seasonality. Similarly, we observed low nematodes load when high MV seroprevalences occurred

(chapter 2). This fact supports the aforementioned idea that Th1/Th2 dichotomy is not complete. Moreover, Chylinski et al. (2009) suggested that rabbits' acquired immunity can regulate nematodes density by controlling directly and indirectly their growth and fecundity. As rabbits mature, the immune response is even more effective and therefore this may decrease intensities of nematode infections.

As noticed in **chapter 2**, seasonal dynamics of parasites were particularly distinctive. The highest intensities of parasites (i.e: coccidia and nematodes) were detected during the breeding season just preceding the peak of high population abundance. Essentially, when such low rabbit densities occurred, MV and RHDV seroprevalences remained at their lowest values. This is in agreement with García-Bocanegra et al. (2011) and Villafuerte et al. (2017) that indicated that antibody prevalence increased in parallel with population density. Once the reproduction takes place, the occurrence of myxomatosis and RHD outbreaks are registered as a result of the recruitment of susceptible young rabbits. At this point, when parasite loads dropped to its minimum and population densities experienced a maximum peak, we observed the highest MV and RHDV seroconversion rates.

As a general tendency, seroconversion to seropositivy was more likely than the reverse (**chapter 1**). Nevertheless, as we suggested previously seropositivy did not confer high survival in all cases. Rabbits seropositive for MV experienced considerable higher survival rates than seronegatives while RHDV seropositivity was never related to an increase in rabbit survival (**chapter 1**). In this sense, effects derived from the haemorrhagic syndrome are likely to overbalance beneficial effects derived from immunity and may lead rabbits seropositive for RHDV to high mortality rates (**chapter 1**).

Negative effects of seropositivity were even more patent among young rabbits that showed low survival rates (both seropositives for MV and also seropositives for RHDV). In this regard, the production of antibodies would be clearly detrimental for young rabbits since their immune system is not fully mature. Definitely, juveniles are the most susceptible age class to diseases as confirmed by García-Bocanegra et al. (2011) and Villafuerte et al. (2017) who revealed that MV and RHDV seroprevalences were lower among young rabbits.

Epidemiologically, age is a key factor because as rabbits get older they are subjected to contact an increasing number of pathogens (i.e: MV, RHDV, coccidia and nematodes). As a consequence of the infections they face during their life-time, the level of antibodies is considerably higher in adult rabbits than in juveniles. Actually, results from **chapter 4** supported this point since we observed age was related positively to MV seropositivity and also dual seropositivity (MV and RHDV).

Otherwise we did not find any association between RHDV seropositivity. This fact is likely to be associated to the virus lethality with the most pronounced impact in early age classes that would prevent them from reaching adulthood. Naturally, wild rabbits infected with pathogens (i.e: MV, RHDV, coccidia and/or nematodes) undergo dramatic changes in behaviour, nutritional condition and physiology. Such changes are the result of pathogens-derived impact and also the ability of the individuals to compensate for these damaging effects that influence body condition (i.e: body weight, growth rate), capacity for reproduction and hence rabbits' fitness (Reglero et al., 2007; Henzell et al., 2008).

As disease outbreaks in wild animals are ephemeral events, the detection of new outbreaks (in the wild) is a really challenging task. However, most recent studies are aimed at detecting prior exposure to specific pathogens (Artois et al., 2009). One

feasible approach is to use chemical compounds from blood or tissues as biomarkers (i.e: antibodies, blood biochemical parameters and/or OS markers) (Franzmann and Schwartz, 1988; Hellgren et al., 1989; Schroeder, 1987; Hellgren et al., 1993;Milner et al., 2003). The levels of these compounds would indicate potential physiological or biochemical changes likely linked with different stages of infection. In this respect, the main advantage of this approach is that it provides epidemiologically useful information to detect disease events within wild populations.

In line with this, we assayed several biochemical parameters **(chapter 3)** that are considered good proxies of physiological condition because they indicate organ function, protein metabolism and nutritional status (Harder and Kirkpatrick, 1994; Stirrat, 2003). Additionally, in **chapter 4** we also measured OS markers which are widely used to assess physiological cost of infection in animal health (Lykkesfeldt and Svendsen, 2007; Castillo- Rodriguez et al., 2011).

As described before, seasonality was evidenced with a maximum peak of parasites that accorded with low rabbit abundances and also low seroprevalence rates. In this period we mostly recorded seronegative rabbits but once new individuals were born, viral outbreaks appeared consequently and the number of seropositive rabbits was largely increased. Here, high seroconversion rates to seropositive status seemed to be associated to an alteration in the level of transaminases (ALT, AST, and GGT) although we only found low concentrations of GGT in rabbits with dual seropositivity (seropositives for both MV and RHDV). Such a decrease in GGT levels is indicative of an impaired hepatic function that is normally observed in rabbits infected with MV and/or RHDV. Furthermore, AOX markers apparently seemed unaffected but rabbits with dual serostatus (seropositives for both MV and RHDV) showed elevated MDA concentrations that is a distinct sign of substantial oxidative damage.

Presumably, a large amount of compounds are susceptible to be altered in wild rabbits during disease processes. Notwithstanding, they may result unnoticed because such changes may be weak and ephemeral in the wild and, it could also occur that other factors (i.e: environmental factors) are likely to mask the real impact on OS parameters (Ferreira et al., 2004; Wobeser, 2007).

Besides, myxomatosis and RHD are now enzootic in the IP and have reduced their severity, so physiological responses to pathogens might be decreasing also in intensity. All this goes to show that despite the recent surge of interest in the use of these biomarkers (i.e: blood biochemistry and OS makers) to assess the impact of wildlife diseases, further research remains to be done to disentangle sources of variation likely to affect these potential physiological indicators.

To conclude, it is worth to highlight the heterogeneous characteristic patterns present in the enclosures studied here. It provides a clear evidence of interactions with other environmental factors and also marked variability within individual-level in immune and physiological responses in accordance with different life-history traits (e.g., developmental, physiological, genetic, and immunological features). All of that certainly contributed to the distinctive seasonality we reported in the studied enclosures and should be largely considered to get better comprehensive understanding of disease dynamics in our study area.

CONCLUSIONS

I. Seropositivity may affect rabbits' survival differently as a consequence of a trade-off among several factors such as: 1) protective effect of antibodies 2) immunosuppressive syndrome and, 3) virus lethality. Rabbits seropositive for myxoma virus (MV) had either a positive or a negative effect on survival that was likely dependent on the interaction with other factors (e.g. physiological condition). Whereas seropositives for rabbit haemorrhagic disease virus (RHDV) did not experience higher survival, the haemorrhagic syndrome of RHDV would produce higher mortality rates among seropositive rabbits.

II. As a general pattern, seroconversion rates from seronegative (status) to seropositive (status) occurred more often than the reverse among the studied populations. This fact supports the endemic character of myxomatosis and rabbit haemorrhagic disease (RHD) in the study area.

III. Th1/Th2 immunological dichotomy seems to not be completely polarized since we found higher MV seroprevalences when low coccidian and nematode loads occurred. And loads of nematodes and coccidia did not explain seasonal RHDV seroprevalence. Parasite dynamics showed high variability likely due to: 1) different climatic conditions, 2) variations in wild rabbit populations, 3) differences in diversity and richness of intermediate hosts and, 4) influence of environmental factors.

IV. Given the strong relationship between coccidia and MV infections, the control of coccidian loads in the enclosures may minimize the impact of MV outbreaks in the populations of study.

V. The current enzootic character of myxomatosis and rabbit haemorrhagic disesase (RHD) in the study area can reduce the severity of these diseases within wild rabbit populations, therefore, alterations in the biochemistry of the individuals might result unnoticed as a consequence of attenuated physiological responses against these pathogens.

VI. Biochemical parameters here used did show poor sensitivity to detect any physiological alteration in rabbits. The use of alternative indicators such as markers of oxidative stress that could incorporate more and precise information is recommended.

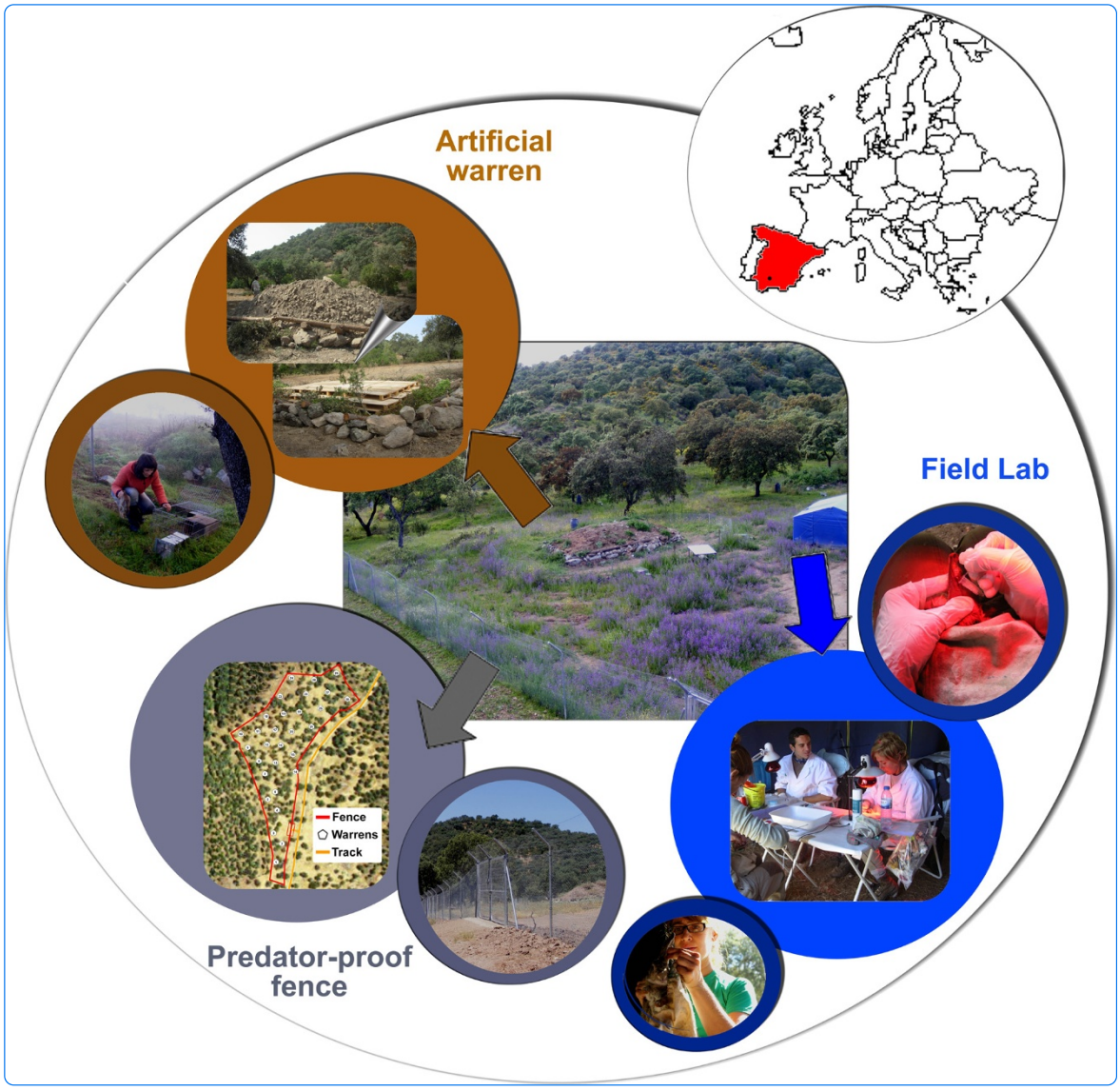
VII. While any association between antioxidant (AOX) and serostatus markers was observed, high concentrations of MDA detected among seropositive rabbits showed a clear evidence of oxidative damage. As an end product of lipid peroxidation, MDA appears to a great extent permanent in the organism. Therefore the use of this marker is a highly reliable tool for medium-term monitoring in wild rabbit populations.

VIII. Generally body condition was positively associated to seropositivity ; rabbits seropositive only for RHDV and rabbits seropositive for both viruses were in better condition than seronegative rabbits. However, this was not the case for rabbits seropositive for MV.

IX. Despite the populations of study experienced similar climatic and management conditions we reported heterogeneous patterns as a result of the interaction with other environmental factors and also due to the variation within individual-level in immune and physiological responses connected to different life-history traits (e.g., developmental, physiological, genetic, and immunological features).

APPENDIX

VETERINARY RESEARCH



Multi-event capture–recapture modeling of host–pathogen dynamics among European rabbit populations exposed to myxoma and Rabbit Hemorrhagic Disease Viruses: common and heterogeneous patterns

Santoro *et al.*

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VETERINARY RESEARCH

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Multi-event capture–recapture modeling of host–pathogen dynamics among European rabbit populations exposed to myxoma and Rabbit Hemorrhagic Disease Viruses: common and heterogeneous patterns

Simone Santoro[1†], Isa Pacios[2†], Sacramento Moreno[2], Alejandro Bertó-Moran[2] and Carlos Rouco[2,3*]

Abstract

Host–pathogen epidemiological processes are often unclear due both to their complexity and over-simplistic approaches used to quantify them. We applied a multi-event capture–recapture procedure on two years of data from three rabbit populations to test hypotheses about the effects on survival of, and the dynamics of host immunity to, both myxoma virus and Rabbit Hemorrhagic Disease Virus (MV and RHDV). Although the populations shared the same climatic and management conditions, MV and RHDV dynamics varied greatly among them; MV and RHDV seroprevalences were positively related to density in one population, but RHDV seroprevalence was negatively related to density in another. In addition, (*i*) juvenile survival was most often negatively related to seropositivity, (*ii*) RHDV seropositives never had considerably higher survival, and (*iii*) seroconversion to seropositivity was more likely than the reverse. We suggest seropositivity affects survival depending on trade-offs among antibody protection, immunosuppression and virus lethality. Negative effects of seropositivity might be greater on juveniles due to their immature immune system. Also, while RHDV directly affects survival through the hemorrhagic syndrome, MV lack of direct lethal effects means that interactions influencing survival are likely to be more complex. Multi-event modeling allowed us to quantify patterns of host–pathogen dynamics otherwise difficult to discern. Such an approach offers a promising tool to shed light on causative mechanisms.

Introduction

Emerging and re-emerging infectious diseases present one of the most pressing issues facing wild vertebrate populations in the 21st century [1]. However, relatively little is yet known about the exposure dynamics of infectious agents in their individual hosts, and what determines their impact on life-history traits such as survival [2]. A comprehensive understanding of the ecological processes influencing pathogen dynamics in natural host populations is of crucial importance to predicting both the dynamics of infectious diseases and the risks that they may entail for animal populations [3,4].

The European wild rabbit (*Oryctolagus cuniculus*) and the two main viral diseases that affect them (myxomatosis, and Rabbit Hemorrhagic Disease) represent an important system for addressing wildlife eco-epidemiological issues. European rabbits are a multifunctional keystone species; not only do they alter plant species composition and vegetation structure, but they also represent the bulk of the diet of a wide variety of Iberian predators [5]. These include the Iberian lynx (*Lynx pardinus*) and the Spanish imperial eagle (*Aquila adalberti*), both seriously threatened [5-7]. The arrival of myxoma virus (MV) and Rabbit Hemorrhagic Disease Virus (RHDV) on the Iberian Peninsula has caused a marked decline in rabbit populations over the last 50 years [8-11], threatening further

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population losses among their predator species [12,13]. In an attempt to prevent local extinction in its native range, and to avoid predator co-extinctions, the European rabbit has been made a conservation priority in both Spain and Portugal [14-17]. In addition the loss of rabbits, considered a pest species across most of their introduced range [18], has in some areas of the Iberian Peninsula caused large-scale economic loss and environmental degradation (e.g. [19,20]).

In spite of the ecological and economic relevance of these issues, disease surveillance still broadly uses simple counts of infected and uninfected animals, although more accurate statistical tools that account for imperfect detection are now available (see [21] for a review of capture–recapture modeling in epidemiology). A few studies have used simple Cormack–Jolly–Seber (CJS) capture–recapture models to investigate MV and RHDV effects on rabbit population dynamics (e.g. [22,23]). As an extension of single-state (CJS) models, multi-state capture–recapture models allow the direct estimation and testing of hypotheses about seroconversion rates and state-specific survival rates [24-26]. However, assessment of the infectious status of all captured individuals is often unfeasible, and some data rearrangement (e.g. data-censoring) is usually required for the application of multi-state models (reviewed in [27]). To avoid these limitations, multi-event capture–recapture models have been recently developed [28] allowing uncertainty in state assessment to be modeled. Here we apply this novel approach to test hypotheses about the effects on survival of, and the dynamics of host immunity to, both MV and RHDV (which occur naturally in the study area). As a general prediction, based on previous studies, we expected MV and RHDV seropositives to have higher survival rates than seronegatives (e.g. [29-31]). In addition, we aimed to do the following: (*i*) estimate monthly survival rates dependent on antibody status, age (juveniles *vs.* adults) and sex, (*ii*) assess monthly seroconversion rates (from seropositive to seronegative, and vice versa) with respect to both diseases according to hosts' age and sex, (*iii*) quantify the dynamics of seroprevalence to each virus over time, expressed as the seropositive probability, and (*iv*) examine relationships between population size and seroprevalence.

Materials and methods

Ethic statement

All animal manipulations reported in this paper were made in accordance with Spanish and European regulations (Law 32/2007, R.D. 1201/2005 and Council Directive 2010/63/EU).

Study area and sampling

The study was carried out in the southwestern Iberian Peninsula (Hornachuelos Natural Park; 37°49′ N, 5°15′ W; 100–700 m elevational range), where the climate is Mediterranean with hot, dry summers and cool, wet winters [32]. A rabbit breeding program was implemented in the area in 2008 for conservation purposes; the main objective was to increase rabbit abundance in the area to enhance survival of threatened predators. Three enclosures (E1, E2, E3; about 4 ha each) were built as breeding zones for rabbits, being surrounded by 2.5-m-high chain-link fence to both prevent rabbit dispersal and exclude terrestrial predators [33]. Each enclosure contained 30 regularly distributed artificial warrens, water and pellet food were supplied *ad libitum*, and grass was sown to increase the availability of fresh food.

From May 2008 to April 2010, nine one-day live-trapping sessions were carried out at each enclosure. Time-intervals between capture sessions varied slightly across enclosures and through the time period; on average a capture session was performed every 2.9 months (SE: 0.18). Live rabbits were caught in cage traps placed surrounding each warren as described in [34], with 50–60% of rabbits from each warren caught at each session [35]. Captured animals were handled at the trap-site, being marked with a numbered ear tag and having their sex and weight recorded. Females and males weighing more than 750 g and 850 g respectively were considered adults [36-38] (see Additional file 1 for details on sex and age population structures). Blood samples (1–2.5 mL) were collected from 1125 (17%) of 6605 captured animals over the course of the study. Details of the serology protocols employed are given in [34]. For both viruses, we considered seropositive those individuals with antibody concentrations sufficient to confer protection against disease as resulting from the ELISA test Information brochure (Ingezim Rabbit 1.7., Ingenasa Laboratory, Madrid, Spain). The probability of either false-positive or false-negative diagnoses under this paradigm are negligible ($P < 0.02$; [34]) making state misclassification unnecessary to be modeled. Furthermore, since antibodies against both viral diseases are detectable in blood for a short time (8–13 days) after exposure to either pathogen [39], seroprevalence to MV or RHDV provides a reliable insight into the epidemiological dynamics of the two diseases.

Multi-event capture–recapture analyses

Capture–recapture sessions were not synchronized among the three fenced areas (E1–E3), for logistic reasons. As a result, we ran separate analyses for each enclosure and disease agent (MV and RHDV). With these populations serving as breeding zones for restocking purposes, random samples of captured individuals were periodically removed during capture sessions (removals of individuals is coded in the data sets and does not affect estimation of parameters). Sample sizes for capture–recapture analyses

(i.e. number of captures minus number of removals) were 2573, 1771 and 1400 for E1–E3 respectively. Numbers of animals captured per trapping session, divided by the surface of the trapped enclosure, were used as indices of rabbit density [35] for each capture session and enclosure.

Goodness of fit

Since no goodness of fit (GOF) is available for multi-event models, for each population we used U-CARE 2.3.2 [40] to test the fit of the Cormack–Jolly–Seber (CJS) model that accounted only for time variation in survival and capture probabilities. The CJS model is therefore more restrictive than those fitted for testing hypotheses that do account for the effect of serological status on recapture and transition probabilities. Hence, this approach is conservative given that if the CJS model adequately fits the data, then the multi-event models are also expected to fit. For each population/GOF analysis, we defined four groups according to age at first capture (adult vs. juvenile) and gender. U-CARE allows testing for specific lack of fit due to a transience effect (i.e. a higher than expected presence of individuals showing up only once; test component 3.SR) and/or to trap-dependence (i.e. capture probability depending on the fact they were captured or not; test component 2.CT). When the overall (all the groups and components together) goodness-of-fit tests were significant, sources of extra-binomial variation were accounted for in the multi-event global models by including transience and/or trap dependence (according to the output of tests 3.SR and 2.CT on each specific group). Over-dispersion factors (*c-hat*) were then calculated as the ratio between the sum of χ^2 values and degrees of freedom of the non-significant test components [41].

For E1, the global goodness-of-fit test indicated lack of fit of the CJS model ($\chi^2 = 112.47$; d.f. = 87; $P = 0.034$), detecting "trap-happiness" among both adult males and females ($P = 0.01$ and < 0.01 respectively). For E2 and E3, there was no evidence of lack of fit ($\chi^2 = 73.53$; d.f. = 88; $P = 0.87$, and $\chi^2 = 73.53$; d.f. = 88; $P = 0.8$, respectively). We thus modeled trap-dependence among adults for E1 (see [42] and Additional file 2 for details on the probabilistic framework used). Correction for over-dispersion (*c-hat* = 1) was not needed for any analysis. Both survival and seroconversion rates have probably varied throughout the study period. However, because of limited sample sizes, and because our primary interest was in the net immunological effects on rabbit dynamics, we chose not to include a time effect on Survival and Seroconversion parameters.

Multi-event design

During each field session (excluding the fifth, for logistical reasons) a variable and random sample of captured individuals were blood-sampled (regardless of sex, age, and encounter history) and their MV and RHDV immunological statuses (seropositive or seronegative) were assessed. To account for uncertainty in state-assessment when an individual is not bled (*sensu* "partial observation"; [27]) we used multi-event capture–recapture models [28] in E-SURGE 1.8.5 [43]. Unlike traditional methods for handling partial information on states, like data censoring or the extra-state approach [26], capture records in the multi-event framework are defined as events (i.e. reflecting the way the underlying biological states are observed in the field). It is therefore possible to define a specific event to record the capture of an individual whose state is unknown. Here we considered four events (not seen, 0; seen, bled and assessed as seronegative, 1; seen, bled and assessed as seropositive, 2; seen and not bled, 3) and three possible states (dead, †; alive seronegative, SN; alive seropositive, SP). A slightly different set of states was used in models accounting for trap-dependence (details on the probabilistic framework are given in Additional file 2).

Multi-event models include three parameter types, Initial State (related to the probability of being in some specific state when first captured), Transition (related to the probability of transition between states) and Event (related to the probabilities of being re-sighted according to the event-mediated information on states). We decomposed Transition into two steps: Survival (the survival probability) and, conditional on still being alive, Seroconversion (the seroconversion probability). Event was decomposed into two steps: Capture (accounting for recapture probability) and, conditional on being captured, State Assignment (accounting for the probability the immunological status was assessed). In this study, Initial State estimates the probability one first-captured individual is seropositive. Therefore, by assuming that the probability of first captured individuals being seropostive reflects the percentage of seropositive individuals in the population (but see [44,45] for a discussion on this), Initial State can be a proxy for the seroprevalence in each population.

We ran six analyses, one for each population–disease combination. We used QAICc values [46], to test for effects of immunity, age and sex on both rabbit survival and seroconversion rates (from seropositive to seronegative, and vice versa). Since populations were closed to immigration and emigration, survival rates referred to real survival rates [47]. We assumed that time intervals were short enough that multiple transitions between serological states were unlikely to occur between two consecutive sessions and no bias was expected on seroconversion estimates [48]. In E-SURGE we marked the "uneven time intervals" option to allow monthly estimates of both survival and seroconversion probabilities

even though intervals between capture sessions were not on a monthly basis. We also used the best model from each analysis to test the effect of population density on Initial State (seroprevalence). For each analysis, we computed the significance and percentage of Initial State variation explained by density using analysis of deviance (ANODEV) [49]. This procedure compares the deviance and number of estimable parameters of three models identical except for the parameter of interest (Initial State in this case) which is: (*i*) constant, (*ii*) full-time dependent, or (*iii*) dependent on density.

Model selection

Based on preliminary model exploration, Initial State and State Assignment depended on, respectively, time and time-by-immunological status and were not further modeled. The other parameters of the global model accounted for these effects: (*i*) Survival on age-by-sex-by-immunological status, (*ii*) Seroconversion on sex-by-immunological status; Capture on sex plus age-by-immunological status-by-time (in E1 also on trap-response).

For each population–disease combination, we first modeled recapture probabilities. The structure for recapture probabilities was then fixed as per the model with the lowest QAICc value, and Survival and Seroconversion probabilities were modeled independently. While we modeled Survival we kept the most parameterized structure for Seroconversion, and vice versa. For each parameter we considered a set of candidate models made of models nested to the global model. To keep the number of tested models as low as possible [46], we only considered interactive effects for parameters whose time-variation was not modeled (i.e. Survival and Seroconversion).

A final set of models combined the best structures for both Survival and Seroconversion (lowest QAICc when modeled independently) ([50], for a similar approach). Hence we used this set of models to compute, for each parameter, model-averaged estimates from models lying within 2 Δ of the best model [46].

Results

Myxoma virus and survival

The relationship between MV seropositivity and survival varied among populations (Table 1, Figure 1, and Additional file 3 for numerical values of parameters). In E1, rabbit survival was variable among the age and sex classes but not between seropositives and seronegatives. In E2, MV-seropositive juveniles appeared to have lower survival rates than seronegatives (on average 25.1% lower, hereafter percentage differences refer to point estimates) but estimates were very imprecise; the same pattern was more evident among adult females (seropositive survival 9.6% lower) but the opposite trend was found among adult males (seropositive survival 6.8% higher). In E3, seropositives had higher survival rates than seronegatives in all age and sex classes (8.2% higher).

Table 1 Myxoma Virus model-selection best models

Enclosure	Parameter	Model effects	np; Dev; $QAIC_c$; w_i
E1	Survival	Age*sex	58; 6694.55; 6813.05; 0.66
		Age	56; 6700.78; 6815.10; 0.23
		Age*immun	58; 6699.49; 6817.99; 0.06
	Seroconversion	Sex*immun	62; 6691.23; 6818.08; 0.92
E2	Survival	Age*sex*immun	53; 4733.87; 4053.88; 0.88
		Age*immun	49; 4750.48; 4059.29; 0.06
	Serocoversion	Sex*immun	53; 4733.87; 4053.88; 1
E3	Survival	Immun	48; 3664.89; 3764.20; 0.69
		Age*sex*immun	54; 3654.65; 3766.85; 0.18
		Sex*immun	50; 3664.83; 3768.43; 0.08
	Seroconversion	Immun	52; 3656.86; 3764.76; 0.71
		Sex*immun	54; 3654.65; 3766.85; 0.25

np, number of parameters; Dev, Deviance; $QAIC_c$, Quasi-Akaike Information Criterion corrected for over-dispersion; w_i, Akaike weight (support of the current model with respect to the candidate set of models). Model notation: immun, the immunological status: for survival it means a different survival rate according to immunological status whereas for seroconversion it means that seroconversion rate from seropositive to seronegative is different than seroconversion from seronegative to seropositive; *age*, juveniles *vs*. adults; *sex*, females *vs*. males.
For each analysis, only models accounting for more than 90% of cumulative Akaike weights are reported.

Rabbit hemorrhagic disease and survival

The relationship between RHDV seropositivity and survival also varied among populations (Table 2, Figure 1, and Additional file 3). However, there was a general pattern of seropositives tending to have lower survival than seronegatives. In E1, there was no clear relationship between RHDV seropositivity and either juvenile or adult female survival, whereas it was related to lower survival (12.6%) in adult males. In E2, seropositivity was related to lower juvenile survival (20%), but no relationship was observed for adults. In E3, seropositive juveniles appeared to survive less (7.1%) than seronegatives, whereas no clear effect was found for adults.

Myxoma virus seroconversion

Overall, the probability of becoming MV seropositive was higher than that of becoming seronegative (Table 1, Figure 2, and Additional file 4 for numerical values of parameters). In E1, both males and females became seropositive at a faster rate than the reverse; for females the probability of becoming seronegative was null suggesting that female rabbits once seropositive remain so. Males appeared to become seropositive at a faster rate than females. In E2, females became seropositive at a faster rate than the reverse, and faster than males. In E3, the

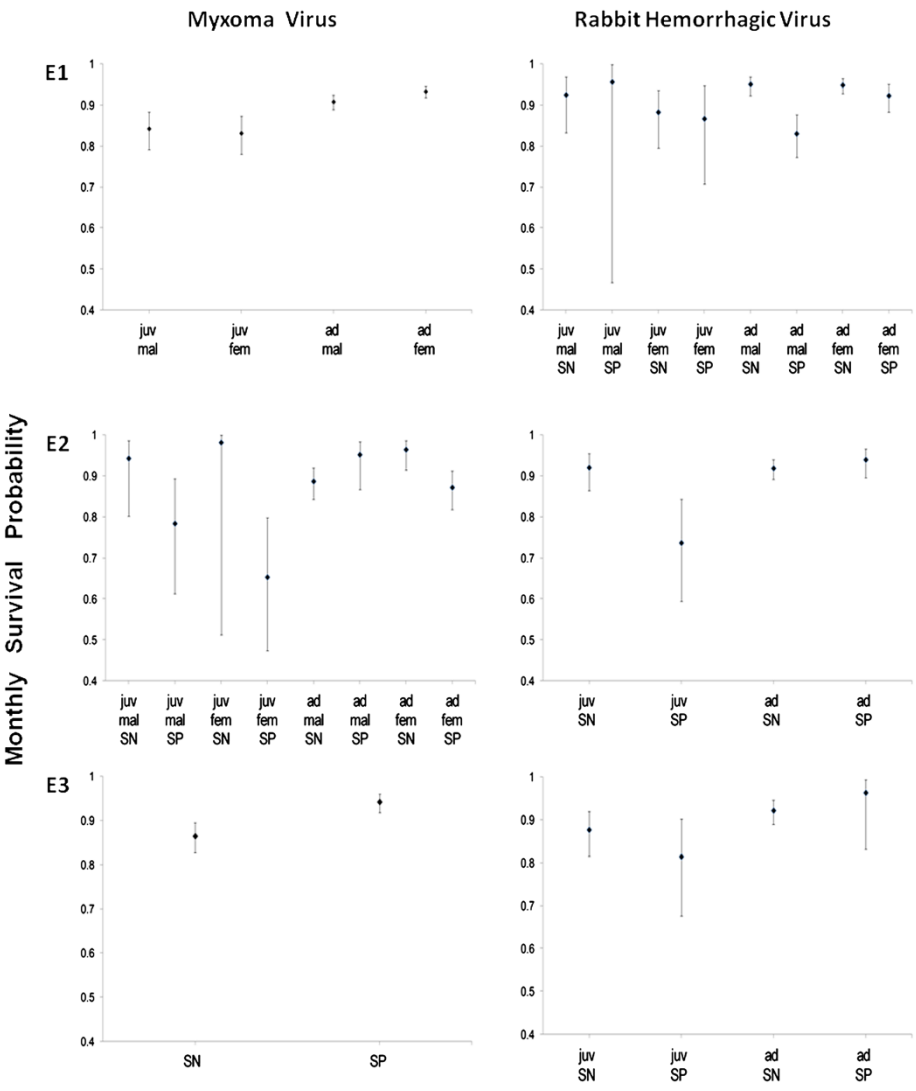

Figure 1 Average monthly survival probability according to serological status (to MV and RHDV), age and gender in the three enclosures (E1, E2, and E3). Only estimates related to effects selected in models with lowest QAICc are shown (e.g. if sex effect on survival was found to be negligible by model selection, one common estimate is shown for both males and females). Estimates with 95% confidence intervals are shown. Notation: juv, juvenile; SN, seronegative; SP, seropositive; mal, males; fem, females.

probability of becoming seropositive was the same for both genders, and the reverse was null.

Rabbit Hemorrhagic Disease Virus seroconversion

We found very different patterns of RHDV seroconversion among enclosures (Table 2, Figure 2, and Additional file 4). In E1, no difference was observed among genders, with all rabbits becoming seropositive at a faster rate than the reverse. In E2, there were no consistent differences in seroconversion rates of males, while females became seropositive at a higher rate than the reverse (and faster than males). In contrast, in E3 both sexes became seropositive at an unusually high rate, and faster than they became seronegative (from model without sex effect: respectively, 0.72, SE: 0.05; 0.14, SE: 0.02).

Seroprevalence and density

Seroprevalence to myxoma and RHD viruses varied across time in all populations (Table 3, and Additional files 5, 6 and 7, respectively), but high levels of uncertainty on several estimates made it difficult to discern time-trends.

Population size in the three enclosures followed similar patterns, being lower during the winter and higher during the spring. The three populations reached maximum peak densities at the same time in May 2010, while minimum densities occurred at different times. Average rabbit densities (no./hectare) per enclosure (± 95% CI) over the whole

Table 2 Rabbit Hemorrhagic Disease Virus model-selection best models

Enclosure	Parameter	Model effects	np; Dev; $QAIC_c$; w_i
E1	Survival	Age*sex*immun	83; 6518.66; 6689.61; 0.73
		Sex*immun	79; 6530.35; 6692.83; 0.15
		Immun	77; 6535.99; 6694.24; 0.07
	Seroconversion	Immun	81; 6519.89; 6686.59; 0.74
		Sex*immun	83; 6518.66; 6689.61; 0.16
E2	Survival	Age*immun	49; 4724.47; 4037.61; 0.62
		Age*sex*immun	53; 4718.07; 4040.71; 0.13
		Age	47; 4733.35; 4040.81; 0.13
		Age*sex	49; 4729.07; 4041.44; 0.09
	Serocoversion	Sex*immun	53; 4718.07; 4040.71; 0.82
		Sex	51; 4726.87; 4043.82; 0.17
E3	Survival	Age*immun	42; 3830.64; 3917.17; 0.42
		Age	40; 3835.32; 3917.62; 0.34
		Immun	40; 3837.85; 3920.15; 0.09
		Age*sex	42; 3834.10; 3920.63; 0.07
	Seroconversion	Sex*immun	46; 3827.61; 3922.65; 0.54
		Immun	44; 3832.35; 3923.13; 0.43

See Table 1 footnote for notations used.

study period were 79.6 ± 53.1, 49.9 ± 31.4, and 52.5 ± 38.5 (for E1–E3 respectively). We observed a marginally significant positive relationship between rabbit density and MV seroprevalence for E1 (slope on logit scale: 1.73; SE: 0.3; *P*: 0.07; 66% variation explained), and a negative such relationship for E3 (slope on logit scale: −1.13; SE: 0.26; *P*: 0.06; 71% variation explained). We also observed a marginally significant positive relationship between density and RHDV seroprevalence in E1 (slope on logit scale: 1.41; SE: 0.17; *P*: 0.06; 73% variation explained).

Recapture probability

Recapture rates varied through time in all analyses (average estimates ± SD from best models of MV analyses: E1, 0.61 ± 0.22; E2, 0.48 ± 0.19; E3, 0.28 ± 0.19). We found either sex, age, or immunological status affected the probability of being recaptured (in E1 trap happiness was also confirmed throughout model selection). However, very heterogeneous causal effects on recapture probabilities were found among and within the rabbit populations.

Discussion

Our study confirms that rabbit populations respond very differently in terms of host survival and epidemiological dynamics when exposed to MV and RHDV, even if geographically close to each other [51,52]. Multiple interacting causes likely explain this heterogeneity. However, some general patterns were observed in our study: (*i*) RHDV seropositivity was never related to increased rabbit survival; (*ii*) seropositivity to both MV and RHDV was negatively related to survival more frequently for juveniles than for adults; and (*iii*) once MV seropositive, rabbits rarely lost such status.

Survival

Our results do not conform to the overall published pattern of MV and RHDV seropositive rabbits always having higher survival rates than seronegatives [29-31]. Only in one case, MV seropositives had considerably higher survival rates than seronegatives. However, it should be noted that our populations were in semi-natural captive conditions allowing high densities, which greatly exceeded wild population densities (e.g. *c*. 3 rabbits/ha for the central Iberian Peninsula; [53]). Therefore our results could be extrapolated to high density scenarios in the wild (e.g. [54,55]).

Seropositivity to MV and RHDV could potentially confer both advantages and disadvantages to rabbits. While it may confer higher immunity to these pathogens, several authors have recognized that (for MV at least) seropositivity has an immunosuppressive effect that may cause higher rates of co-infection with other pathogens [56-58]. With the target organ of RHDV (the liver) being part of the immune system, RHDV may also cause an immunosuppressive syndrome in addition to reduced survival due to hemorrhagic effects [59-61]. Rabbits seropositive to either virus are thus likely to be more susceptible to damage from other infections. A shifting balance of advantages and disadvantages is thus one explanation for contrasting patterns, and is likely influenced by interactions with other environmental, epidemiologic and individual factors. For example, in our population E3 rabbits were in a better physiological status than in the others as a result of availability of higher water quality (e.g. in streams, manuscript in preparation). We suggest that this reduced the negative effect of the MV-seropositive-related immunosuppression, explaining why they had higher survival rates than seronegatives. As another example, RHDV seropositivity never being associated with higher survival rates could indicate that the negative effects of the hemorrhagic syndrome outweighed any beneficial effects. Finally, seropositive juveniles tending to survive less than seronegatives may be explained by the immaturity of the juvenile immune system with full immunity not yet being fully acquired. In light of this, it is possible that negative influences such as immunosuppression and the hemorrhagic syndrome of RHDV had greater influence on juveniles than adults.

Seroconversion

With ongoing persistence 50 and 30 years after the arrival of myxomatosis and RHD respectively on the Iberian

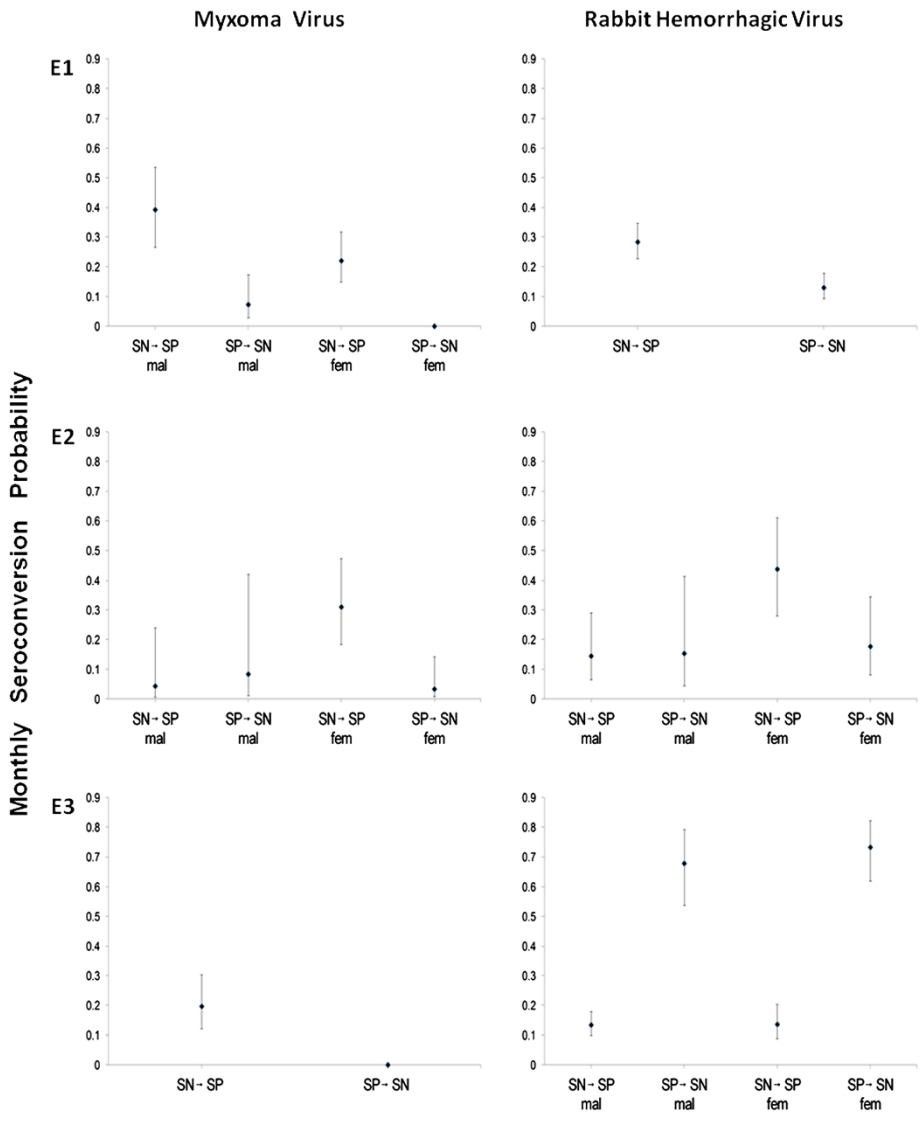

Figure 2 Myxoma and Rabbit Hemorrhagic Disease Virus seroconversion rates in the three enclosures (E1, E2, and E3). Estimates with 95% confidence intervals are shown. Notation: SN ⟶ SP, average monthly rate at which individuals change their immunological status from seronegative to seropositive; SP ⟶ SN, from seropositive to seronegative; mal, males; fem, females.

Table 3 Effect of rabbit density on Initial State (seroprevalence of MV and RHDV)

	Myxo – Prev	RHD – Prev
E1	4.65; 0.07; 0.66	5.11; 0.06; 0.73
E2	0.25; 0.63; 0.03	0.80; 0.4; 0.11
E3	5.01; 0.06; 0.71	2.94; 0.13; 0.42

From left to right: the Fisher–Snedecor statistic ($F_{1,7}$ for Initial State and $F_{1,6}$ for the others), *P*-value (in bold if < 0.1), and R^2. All statistics were computed following the ANODEV procedure. Myxo, myxoma virus; RHD, Rabbit Hemorrhagic Disease Virus; Prev, prevalence.

Peninsula [62], it is reasonable to classify the viruses as endemic. Accordingly, rabbits should gain MV and RHD antibodies as fast as or faster than they lose them. Seroconversion to seropositive status tending to occur at a faster rate than the reverse in our study (and also [62] and [31]) supports this expectation. However, this was not the case for RHDV in E3, where seroconversion to seronegative status occurred at a higher rate. We suggest this result does not disprove the endemic disease behavior hypothesized above, but is driven by asynchrony between population and virus dynamics. In fact, RHDV was found in this population over the 7 years of monitoring (SM, unpublished data), but the population had a delayed breeding season that, differently from in

the other enclosures, occurred after the RHD outbreak. Thus, in E3 the naïve kittens would have lost their maternal antibodies after 2 months [63] and, since they were not exposed to RHDV, a large number of seronegative adults (many of them born the spring just before) would have occurred in the next autumn. This would result in a large number of seronegatives over the study period and explain this seemingly inconsistent result. It should also be noted that, in contrast to general belief [64], the average monthly probability of losing antibodies was not necessarily null for either RHDV or MV. This indicates that rabbits can lose immunity to these diseases (albeit with a very low probability). The effect of sex on the rate at which individuals became seropositive varying among populations further illustrates the varying behavior of these diseases across individuals and populations [51,52].

Antibody prevalence and density

In general, seroprevalence did not follow a consistent pattern either within or among populations for either disease. This was in agreement with previous findings from some authors, stating these diseases behave very differently among populations [51,52], but contrasts with a study in the Canary Islands [65] where they found no difference in RHDV prevalence across four neighboring geographic zones.

Host infection by both viruses also occurs by means of contact with an infected individual. Population size is thus recognized as an important factor promoting MV and RHDV dynamics [52,66-68]. However, even though in some cases a great amount of variation in seroprevalence appeared to be explained by density, in no case did we find this hypothesis was strongly supported (*P*-values were marginally significant at the 0.05 level). The pattern of increasing prevalence with population size observed in E1 for both MV and RHDV was unsurprising. However, we observed no such relationships in E2 and a negative relationship between MV prevalence and population size in E3. This last result may have been caused by a correlation between decreasing density due to gradual habitat degradation (authors personal observation) leading to individuals under nutritional stress being more susceptible to infection.

Conclusion

This is the first multi-event study focusing on MV and RHDV host–pathogen dynamics in rabbit populations. We found that while MV seropositivity had either a positive or negative effect on survival that was likely dependent on interaction with other factors (e.g. physiological condition), the hemorrhagic syndrome caused by RHDV led seropositive rabbits to suffer higher mortality rates. Our study highlights that the host–pathogen dynamics of these viruses are highly variable among populations even when these share similar management and climatic conditions. These findings have important implications for rabbit population management, particularly where their scarcity could compromise ecosystem conservation. Additional well-defined capture–recapture analyses may shed further light on the still many obscure mechanisms driving host–pathogen dynamics (e.g. [69,70]).

Additional files

Additional file 1: Sex and age population structures in each enclosure (E1, E2 and E3). Frequency of males and females, juveniles and adults, as resulting from captured individuals for each session capture in the three enclosures.

Additional file 2: Probabilistic framework of the multi-event analyses. Matrice structure for each parameter (Initial State, Survival, Seroconversion, Capture or Trap-Dependence, and State Assignment) is given and its notation explained.

Additional file 3: Survival estimates with 95% CI for each enclosure (E1, E2 and E3). Survival estimates in the three enclosures as depending on the main effects found through model selection.

Additional file 4: Seroconversion estimates with 95% CI for each enclosure (E1, E2 and E3). Seroconversion estimates in the three enclosures as depending on the main effects found through model selection. No age effect was tested because juveniles remain juveniles only for a short time and no recapture from one individual in juvenile state exists.

Additional file 5: Myxoma virus and RHDV antibody prevalence in enclosure E1. Myxoma virus and Rabbit Hemorrhagic Disease Virus seroprevalences over the study period as proxied by Initial State in enclosure E1. Prevalence estimates refer to the probability one first-captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represents 95% confidence intervals.

Additional file 6: Myxoma virus and RHDV antibody prevalence in enclosure E2. Myxoma virus and Rabbit Hemorrhagic Disease Virus seroprevalences over the study period as proxied by Initial State in enclosure E2. Prevalence estimates refer to the probability one first-captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represent 95% confidence intervals.

Additional file 7: Myxoma virus and RHDV antibody prevalence in enclosure E3. Myxoma virus and Rabbit Hemorrhagic Disease Virus seroprevalences over the study period as proxied by Initial State in enclosure E3. Prevalence estimates refer to the probability one first-captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represent 95% confidence intervals.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CR and SS conceived and designed the study. IP and SM participated in the design of the study. CR, IP, SM and AB assisted in the field with animal handling and collected and processed the samples. AB conducted the serological analysis. SS carried out all statistical analysis. CR, SS and IP drafted the manuscript and SM and AB helped edit the manuscript. All authors read and approved the final manuscript.

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References

1. Dobson AP, Foufopoulos J: **Emerging infectious pathogens of wildlife.** *Phil Trans R Soc B* 2001, **356:**1001–1012.
2. Benskin CMH, Wilson K, Jones K, Hartley IR: **Bacterial pathogens in wild birds: a review of the frequency and effects of infection.** *Biol Rev Camb Philos Soc* 2009, **84:**349–373.
3. Daszak P, Cunningham AA, Hyatt AD: **Emerging infectious diseases of wildlife - threats to biodiversity and human health.** *Science* 2000, **287:**443–449.
4. Chambert T, Staszewski V, Lobato E, Choquet R, Carrie C, McCoy KD, Tveraa T, Boulinier T: **Exposure of black-legged kittiwakes to Lyme disease spirochetes: dynamics of the immune status of adult hosts and effects on their survival.** *J Anim Ecol* 2012, **81:**986–995.
5. Delibes-Mateos M, Delibes M, Ferreras P, Villafuerte R: **Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot.** *Conserv Biol* 2008, **22:**1106–1117.
6. Ferrer M, Negro JJ: **The near extinction of two large European predators: super specialists pay a price.** *Conserv Biol* 2004, **18:**344–349.
7. Delibes-Mateos M, Ferreras P, Villafuerte R: **European rabbit population trends and associated factors: a review of the situation in the Iberian Peninsula.** *Mamm Rev* 2009, **39:**124–140.
8. Calvete C, Pelayo E, Sampietro J: **Habitat factors related to wild rabbit population trends after the initial impact of rabbit haemorrhagic disease.** *Wildl Res* 2006, **33:**467–474.
9. Delibes-Mateos M, Redpath SM, Angulo E, Ferreras P, Villafuerte R: **Rabbits as a keystone species in southern Europe.** *Biol Conserv* 2007, **137:**149–156.
10. Delibes-Mateos M, Farfán MA, Olivero J, Vargas J: **Land-use changes as a critical factor for long-term wild rabbit conservation in the Iberian Peninsula.** *Environ Conserv* 2010, **37:**169–176.
11. García-Bocanegra I, Astorga RJ, Napp S, Huerta B, Carbonero A, Perea A, Arenas A: **Factors affecting the seroprevalence of lagovirus infection in wild rabbits (*Oryctolagus cuniculus*) in Southern Spain.** *Vet J* 2011, **189:**89–94.
12. Moreno S, Villafuerte R, Cabezas S, Lombardi L: **Wild rabbit restocking for predator conservation in Spain.** *Biol Conserv* 2004, **118:**183–193.
13. Moleon M, Sanchez-Zapata JA, Real J, García-Charton JA, Gil-Sánchez JM, Palma L, Bautista J, Bayle J: **Large-scale spatio-temporal shifts in the diet of a predator mediated by an emerging infectious disease of its main prey.** *J Biogeogr* 2009, **36:**1502–1515.
14. Rogers PM: **Predator–prey relationship between rabbit and lynx in southern Spain.** *Terre Vie* 1978, **32:**83–87.
15. Moreno S, Villafuerte R: **Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain.** *Biol Conserv* 1995, **73:**81–85.
16. Palomares F, Gaona P, Ferreras P, Delibes M: **Positive effects on game species of top predators by controlling smaller predator populations: an example with lynx, mongoose and rabbits.** *Conserv Biol* 1995, **9:**295–305.
17. Travaini A, Delibes M, Ferreras P, Palomares F: **Diversity, abundance or rare species as a target for the conservation of mammalian carnivores: a case study in southern Spain.** *Biodivers Conserv* 1997, **6:**529–535.
18. Lees AC, Bell DJ: **A conservation paradox for the 21st century: the European wild rabbit *Oryctolagus cuniculus*, an invasive alien and an endangered native species.** *Mamm Rev* 2008, **38:**304–320.
19. Virgós E, Cabezas-Díaz S, Lozano J: **Is the wild rabbit (*Oryctolagus cuniculus*) a threatened species in Spain? Sociological constraints in the conservation of species.** *Biodivers Conserv* 2007, **16:**3489–3504.
20. Barrio IC, Bueno CG, Tortosa FS: **Alternative food and rabbit damage in vineyards of southern Spain.** *Agric Ecosyst Environ* 2010, **138:**51–54.
21. Cooch EG, Conn PB, Ellner SP, Dobson AP, Pollock KH: **Disease dynamics in wild populations: modeling and estimation: a review.** *J Ornithol* 2010, **152:**485–509.
22. Fordham DA, Sinclair RG, Peacock DE, Mutze GJ, Kovaliski J, Cassey P, Capucci L, Brook BW: **European rabbit survival and recruitment are linked to epidemiological and environmental conditions in their exotic range.** *Austral Ecol* 2012, **37:**945–957.
23. Guitton J-S, Devillard S, Guénézan M, Fouchet D, Pontier D, Marchandeau S: **Vaccination of free-living juvenile wild rabbits (*Oryctolagus cuniculus*) against myxomatosis improved their survival.** *Prev Vet Med* 2008, **84:**1–10.
24. Arnason AN: **The estimation of population size, migration rates and survival in a stratified population.** *Res Popul Ecol* 1973, **15:**1–8.
25. Senar JC, Conroy MJ: **Multi-state analysis of the impacts of avian pox on a population of Serins (*Serinus serinus*): the importance of estimating recapture rates.** *Anim Biodivers Conserv* 2004, **27:**133–146.
26. Faustino CR, Jennelle CS, Connolly V, Davis AK, Swarthout EC, Dhondt AA, Cooch EG: ***Mycoplasma gallisepticum* infection dynamics in a house finch population: seasonal variation in survival, encounter and transmission rate.** *J Anim Ecol* 2004, **73:**651–669.
27. Conn PB, Cooch EG: **Multistate capture-recapture analysis under imperfect state observation: an application to disease models.** *J Appl Ecol* 2009, **46:**486–492.
28. Pradel R: **Multievent, an extension of multistate capture-recapture models to uncertain states.** *Biometrics* 2005, **61:**442–447.
29. Bruce JS, Twigg LE, Gray GS: **The epidemiology of rabbit haemorrhagic disease, and its impact on rabbit populations, in south-western Australia.** *Wildl Res* 2004, **31:**31–49.
30. Calvete C: **The use of immunization programs in wild populations: Modelling effectiveness of vaccination campaigns against rabbit hemorrhagic disease.** *Biol Conserv* 2006, **130:**290–300.
31. García-Bocanegra I, Astorga RJ, Napp S, Casal J, Huerta B, Borge C, Arenas A: **Myxomatosis in wild rabbits: design of control programs in Mediterranean ecosystems.** *Prev Vet Med* 2010, **93:**42–50.
32. Pinilla R, Tamajón R, Muñoz JM: **Vegetación actual**. In *Reconocimiento Biofísico de Espacios Naturales Protegidos*, Parque Natural Sierra de Hornachuelos. Edited by Consejería de Medio Ambiente. Seville, Spain: Junta de Andalucía; 1995:207–247.
33. Rouco C, Ferreras P, Castro F, Villafuerte R: **The effect of exclusion of terrestrial predators on short-term survival of translocated European wild rabbits.** *Wildl Res* 2008, **35:**625–632.
34. Bertó-Moran A, Pacios I, Serrano E, Moreno S, Rouco C: **Coccidian and nematode infections influence prevalence of antibody to myxoma and rabbit hemorrhagic disease viruses in European rabbits.** *J Wildl Dis* 2013, **49:**10–17.
35. Rouco C, Villafuerte R, Castro F, Ferreras P: **Effect of artificial warren size on a restocked European wild rabbit population.** *Anim Conserv* 2011, **14:**117–123.
36. Soriguer RC: *Biología y Dinámica de Una Población de Conejos (Oryctolagus cuniculus, L.) en Andalucía Occidental.* Doñana: Acta Vertebrata 8; 1981.
37. Villafuerte R: **Riesgo de predación y estrategias defensivas del conejo en el Parque Nacional de Doñana**. In *PhD thesis.* Córdoba: University of Córdoba; 1994.
38. Alves P, Moreno S: **Estudo da reprodução do coelho-bravo (*Oryctolagus cuniculus*) em Portugal.** *Rev Florestal* 1996, **9:**149–166.
39. OIE (World Organisation for Animal Health): *Technical disease cards*; 2013 [http://www.oie.int/en/animal-health-in-the-world/technical-disease-cards/]
40. Choquet R, Reboulet A, Lebreton J, Gimenez O, Pradel R: *U-CARE 2.2 User's Manual.* Montpellier, France: CEFE; 2005.
41. Choquet R, Lebreton J-D, Gimenez O, Reboulet A, Pradel R: **U-CARE: utilities for performing goodness of fit tests and manipulating capture recapture data.** *Ecography* 2009, **32:**1–4.
42. Pradel R, Sanz-aguilar A: **Modeling trap-awareness and related phenomena in capture-recapture studies.** *PLoS One* 2012, **7:**1–4.
43. Choquet R, Nogue E: *E-SURGE 1.8 User's Manual. (English edition).* Montpellier, France: CEFE; 2011.

44. Pradel R: **The Stakes of Capture-Recapture Models with State Uncertainty**. In *Modeling Demographic Processes In Marked Populations*. Edited by Thomson DL, Cooch EG, Conroy MJ. New York: Springer; 2009:781–795.
45. Genovart M, Pradel R, Oro D: **Exploiting uncertain ecological fieldwork data with multi-event capture–recapture modelling: an example with bird sex assignment.** *J Anim Ecol* 2012, **81:**970–977.
46. Burnham KP, Anderson DR: *Model Selection and Multimodel Inference: a Practical Information-theoretic Approach*. 2nd edition. New York: Springer; 2002.
47. Lebreton JD, Burnham KP, Clobert J, Anderson DR: **Modelling survival and testing biological hypotheses using marked animals: a unified approach with case studies.** *Ecol Monogr* 1992, **62:**67–118.
48. Zipkin EF, Jennelle CS, Cooch EG: **A primer on the application of Markov chains to the study of wildlife disease dynamics.** *Methods Ecol Evol* 2010, **1:**192–198.
49. Skalski JR, Hoffmann A, Smith SG: **Testing the significance of individual-and cohort-level covariates in animal survival studies**. In *Marked Individuals in the Study of Bird Population*. Edited by Lebreton JD, North PM. Bäsel, Switzerland: Birkhäuser; 1993:9–28.
50. Grosbois V, Tavecchia G: **Modeling dispersal with capture - recapture data: disentangling decisions of leaving and settlement.** *Ecology* 2003, **84:**1225–1236.
51. Parkes JP, Norbury GL, Heyward RP, Sullivan G: **Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997–2001.** *Wildl Res* 2002, **29:**543–555.
52. Fouchet D, Guitton J-S, Marchandeau S, Pontier D: **Impact of myxomatosis in relation to local persistence in wild rabbit populations: the role of waning immunity and the reproductive period.** *J Theor Biol* 2008, **250:**593–605.
53. Fernández de Simón J, Díaz-Ruiz F, Cirilli F, Tortosa FS, Villafuerte R, Delibes-Mateos M, Ferreras P: **Towards a standardized index of European rabbit abundance in Iberian Mediterranean habitats.** *Eur J Wildl Res* 2011, **57:**1091–1100.
54. Moller H, Clapperton BK, Fletcher DJ: **Density of rabbits (*Oryctolagus cuniculus* L.) in the Mackenzie Basin, South Island, New Zealand.** *N Z J Ecol* 1997, **21:**161–167.
55. Mutze G, Kovaliski J, Butler K, Capucci L, McPhee S: **The effect of rabbit population control programmes on the impact of rabbit haemorrhagic disease in south-eastern Australia.** *J Appl Ecol* 2010, **47:**1137–1146.
56. Boag B: **Observations on the seasonal incidence of myxomatosis and its interactions with helminth parasites in the European rabbit (*Oryctolagus cuniculus*).** *J Wildl Dis* 1988, **24:**450–455.
57. Cattadori IM, Albert R, Boag B: **Variation in host susceptibility and infectiousness generated by co-infection: the myxoma-*Trichostrongylus retortaeformis* case in wild rabbits.** *J R Soc Interface* 2007, **4:**831–840.
58. Cattadori IM, Boag B, Hudson PJ: **Parasite co-infection and interaction as drivers of host heterogeneity.** *Int J Parasitol* 2008, **38:**371–380.
59. Mitro S, Krauss H: **Rabbit hemorrhagic disease: a review with special reference to its epizootiology.** *Eur J Epidemiol* 1993, **9:**70–78.
60. Ferreira PG, Costa-E-Silva A, Oliveira MJR, Monteiro E, Águas AP: **Leukocyte-hepatocyte interaction in calicivirus infection: differences between rabbits that are resistant or susceptible to rabbit haemorrhagic disease (RHD).** *Vet Immunol Immunopathol* 2004, **103:**217–221.
61. Ferreira PG, Costa-e-Silva A, Monteiro E, Oliveira MJR, Águas AP: **Liver enzymes and ultrastructure in rabbit haemorrhagic disease (RHD).** *Vet Res Commun* 2006, **30:**393–401.
62. Calvete C, Estrada R, Villafuerte R, Osácar JJ, Lucientes J: **Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits.** *Vet Rec* 2002, **150:**776–782.
63. Calvete C, Estrada R, Lucientes J, Osacar JJ, Villafuerte R: **Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits.** *Vet Rec* 2004, **155:**388–392.
64. Kerr PJ, Hone J, Perrin L, French N, Williams CK: **Molecular and serological analysis of the epidemiology of myxoma virus in rabbits.** *Vet Microbiol* 2010, **143:**167–178.
65. Foronda PR, Figueruelo EO, Ortega AR, Abreu NA, Casanova JC: **Parasites (viruses, coccidian and helminthes) of the wild rabbit (*Oryctolagus cuniculus*) introduced to Canary Islands from Iberian Peninsula.** *Acta Parasitol* 2005, **50:**80–84.
66. Cooke BD, Fenner F: **Rabbit haemorrhagic disease and the biological control of wild rabbits, *Oryctolagus cuniculus*, in Australia and New Zealand.** *Wildl Res* 2002, **29:**689–706.
67. Calvete C: **Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease.** *Conserv Biol* 2005, **20:**1232–1241.
68. Cotilla I, Delibes-Mateos M, Ramírez E, Castro F, Cooke BD, Villafuerte R: **Establishing a serological surveillance protocol for rabbit hemorrhagic disease by combining mathematical models and field data: implication for rabbit conservation.** *Eur J Wildl Res* 2010, **56:**725–733.
69. Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC: **Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates.** *J Anim Ecol* 2011, **80:**1207–1216.
70. Lachish S, Knowles SCL, Alves R, Wood MJ, Sheldon BC: **Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure.** *J Anim Ecol* 2011, **80:**1196–1206.

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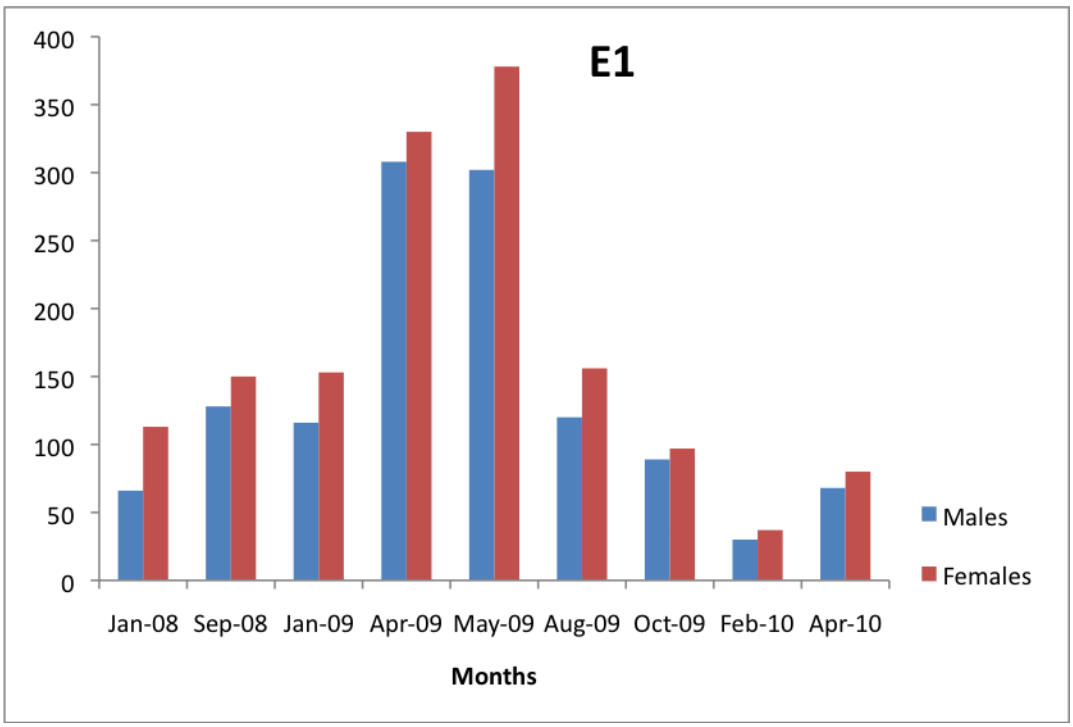
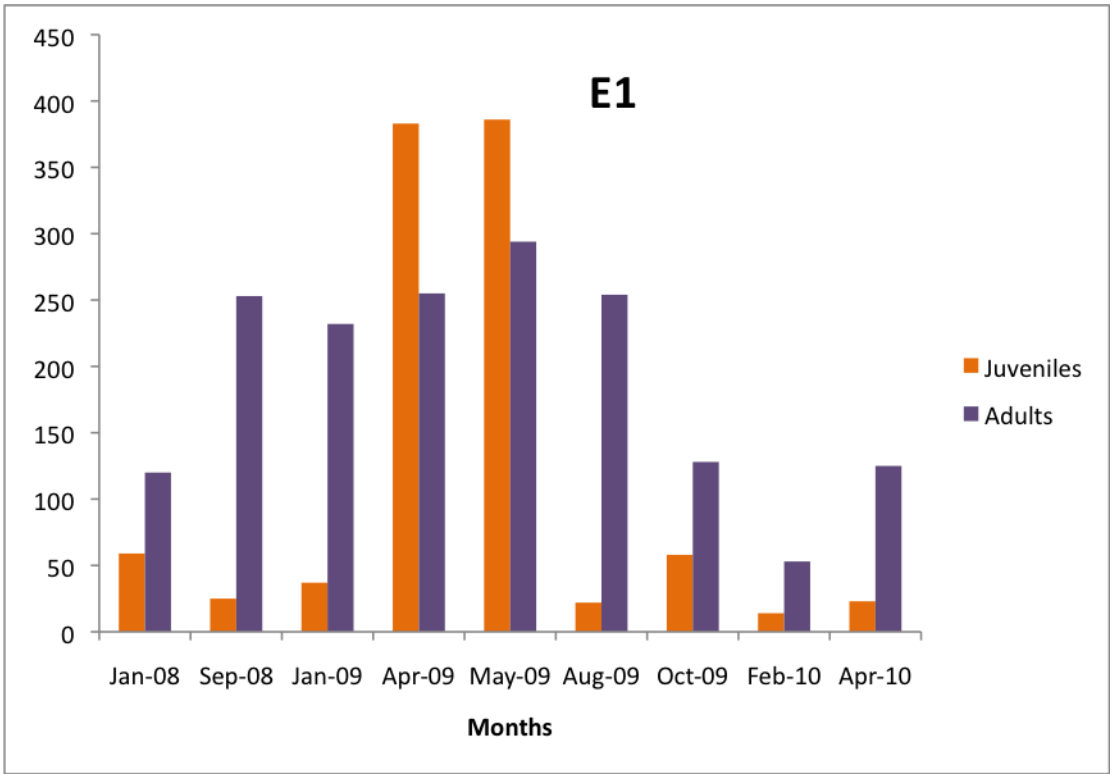
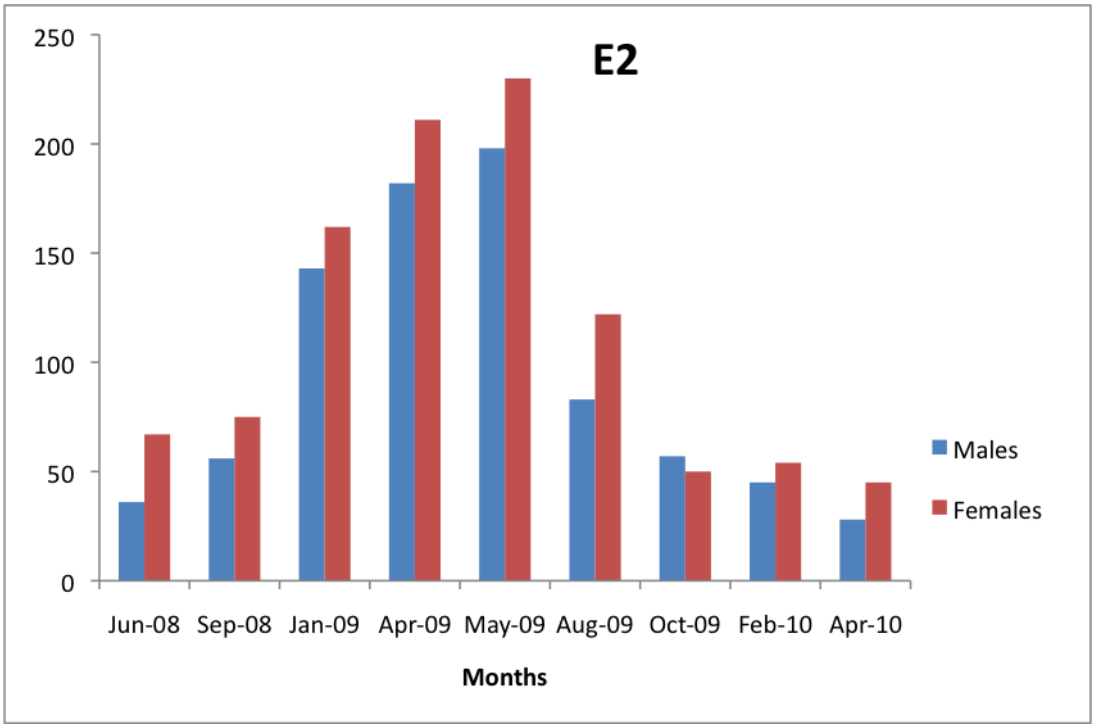
Additional file 1. Sex and age population structures in each enclosure (E1, E2 and E3). Frequency of males and females, juveniles and adults, as resulting from captured individuals for each session capture in the three enclosures.



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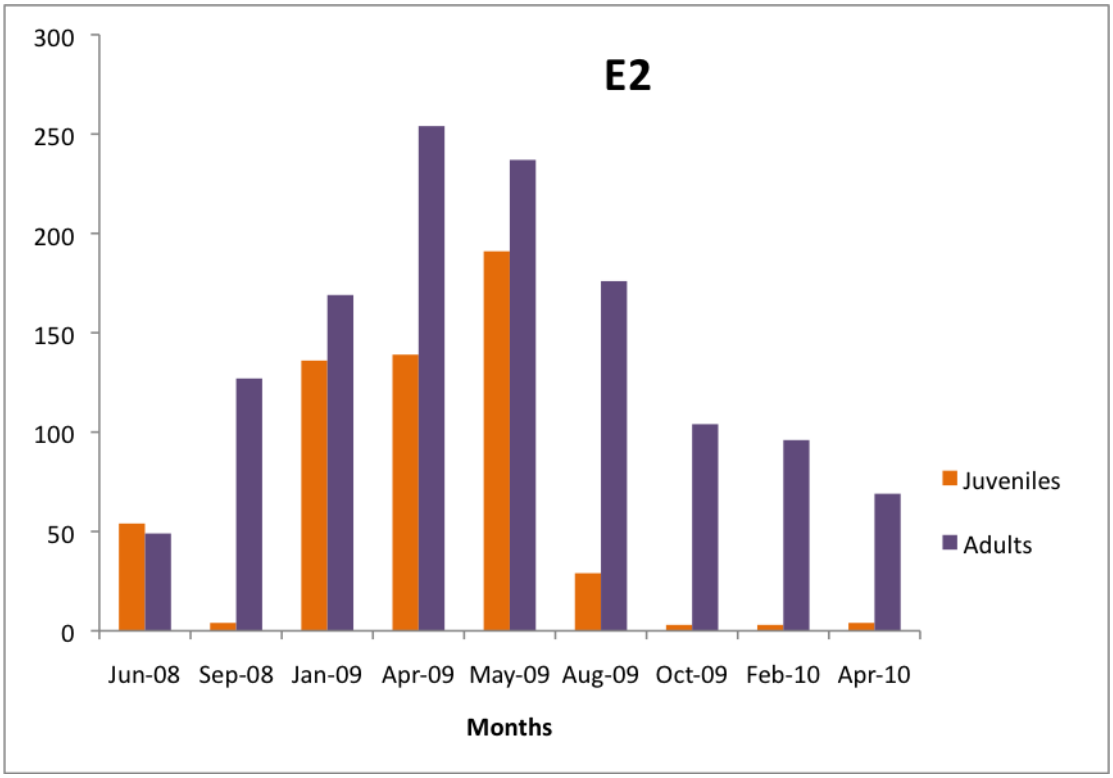
Feb-10
Apr-10
Months
Males
Females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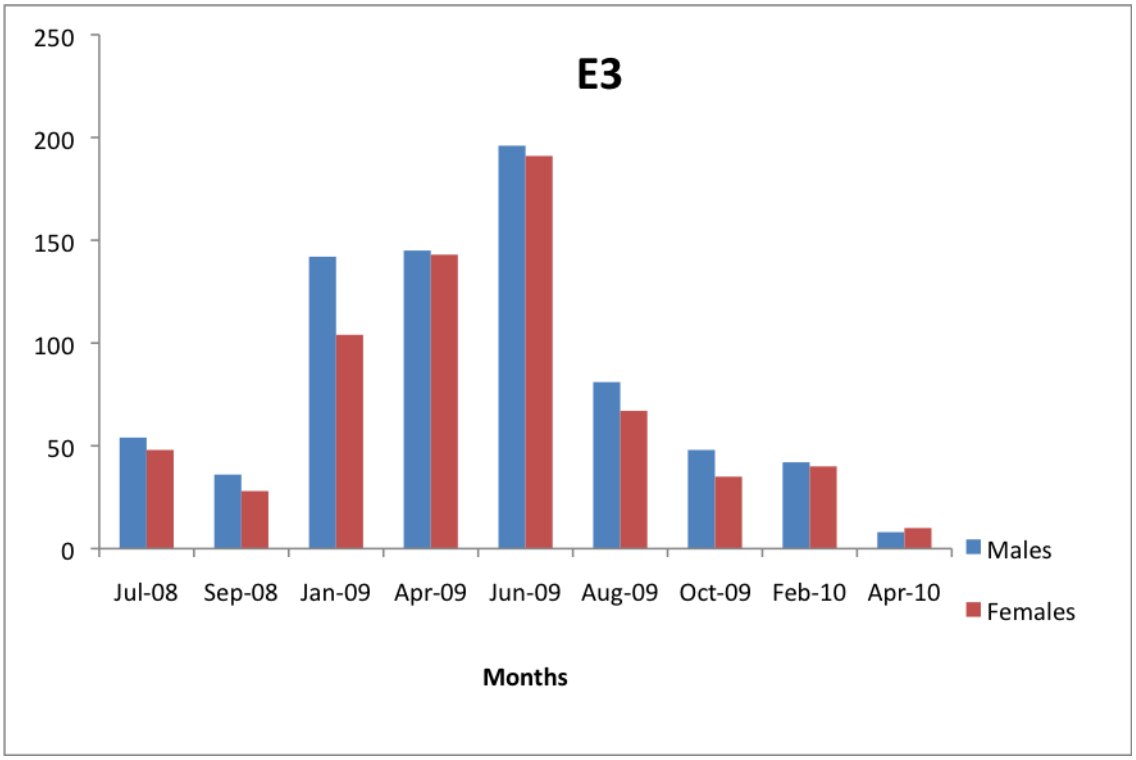
Months
Juveniles
Adults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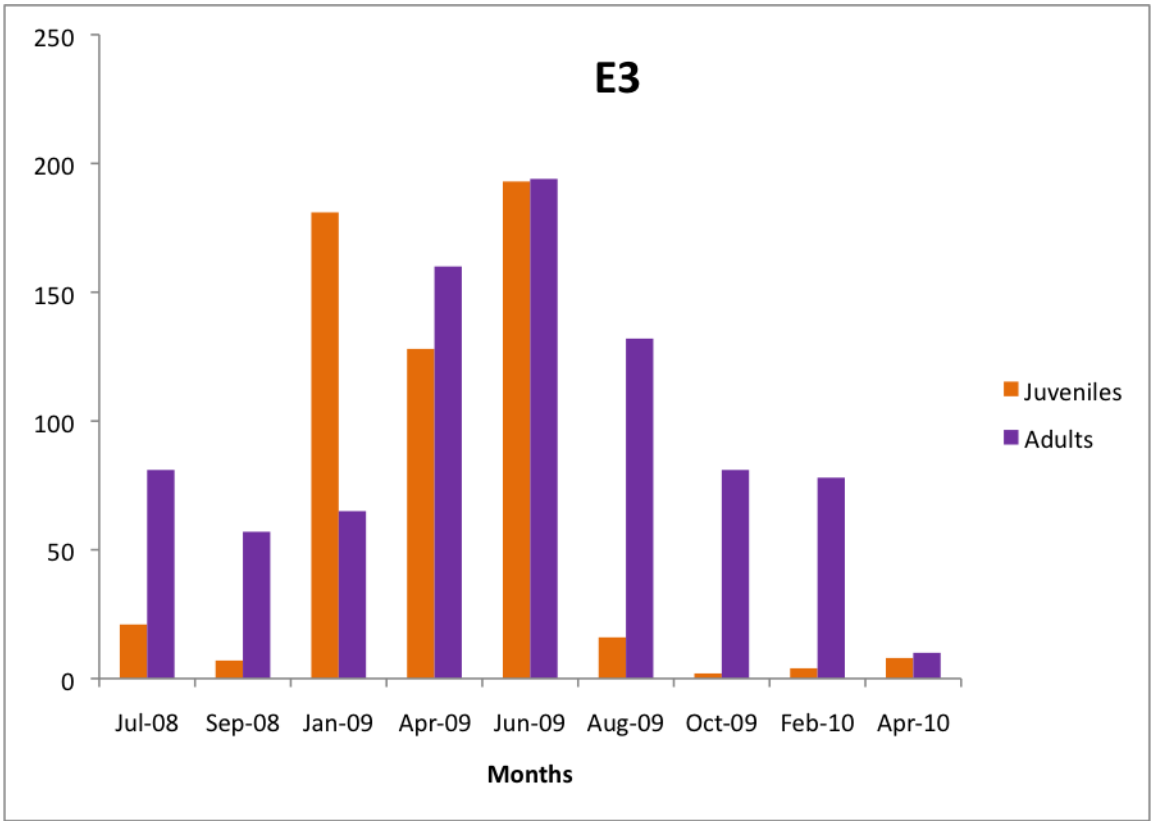
Males
Females
Months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Aug-09
Oct-09
Feb-10
Apr-10
Juveniles
Adults
Months

Additional file 2. Probabilistic framework of the multi-event analyses. Matrice structure for each parameter (Initial State, Survival, Seroconversion, Capture or Trap-Dependence, and State Assignment) is given and its notation explained.

Multievent probabilistic framework of the study

Multievent models combine information from events with the underlying states to estimate probabilities of several parameters. A multievent model accounts for three parameter types: Initial State, State Transition and Event probabilities.

In this study we defined three underlying biological states:
† - Death
SN - Seronegative alive
SP - Seropositive alive

And four events, numbered as they appear in the data set:
0 - Rabbit not captured
1 - Rabbit captured, bled and classified as seronegative
2 - Rabbit captured, bled and classified as seropositive
3 - Rabbit captured, not bled

Groups:

Groups are coded within the "headed format" data file (see [43]) as: (1) Juvenile Male, (2) Juvenile Female, (3) Adult Male, and (4) Adult Female.

INITIAL STATE

This parameter refers to the probability that, an individual is seropositive when first captured. As stated by Pradel [28]: "*The "initial state probabilities", [...] describe the probability that an individual is in one or another state when it is first encountered [...] This is an entirely new type of parameter from multistate models. Dependent on the kind of study, it can be related to the sex-ratio, the prevalence of a disease or the percentage of breeders in the population*".

This parameter here relates to the proportion of seropositives among the new unmarked individuals captured. By assuming that (*i*) the serological status does not seriously affect the probability of being captured, and (*ii*) the sampling scheme has not varied during the study period, this parameter can be related to the seroprevalence of the population (see [28,45] for a discussion on this). By definition, the probability of being first captured as a death individual is zero. Therefore, the initial state probabilities are:

SN	SP
1-π	π

where π is the probability, at a certain occasion *t*, a first captured rabbit has to be seropositive (note that no age or sex effect has been modeled on this parameter).

STATE TRANSITION

This parameter type was divided in two steps. These probabilities are best represented in the form of stochastic matrices with departure states in rows and arrival states in columns.

Step 1 Survival; this step computes the probability that an individual first captured on occasion *t* survives in the interval between *t* and *t* + 1.

	SN	SP	†
SN	φ_{SN}	0	$1-\varphi_{SN}$
SP	0	φ_{SP}	$1-\varphi_{SP}$
†	0	0	1

Where φ_{SN} and φ_{SP} refer respectively to the probability a seronegative or a seropositive has of surviving in the interval after its first capture.

Step 2 Seroconversion (conditional on Survival); this step computes the probability a survived individual has of changing its state between *t* and *t* + 1.

	SN	SP	†
SN	$1-\psi_{SN}$	ψ_{SN}	0
SP	ψ_{SP}	$1-\psi_{SP}$	0
†	0	0	1

Where, conditioned on having survived the current interval, ψ_{SN} and ψ_{SP} refer respectively to the probability a seronegative has to become a seropositive and a seropositive has to become a seronegative.

EVENT

The Event probabilities relate the events to the underlying biological states. This parameter type has been divided into two steps.

Step 1 Capture. This estimates the probability one individual has to be recaptured at *t* + 1.

	not captured	SN captured	SP captured
SN	$1 - \beta_{SN}$	β_{SN}	0
SP	$1 - \beta_{SP}$	0	β_{SP}
†	1	0	0

Where β_{SN} and β_{SP} refer respectively to the probability a seronegative and a seropositive have to be captured.

Step 2 State Assignment (conditional on Capture); this estimates, conditional on being captured, the probability one individual has to be bled when captured. In columns are the events as they are coded in the data set.

	0-not captured	1-captured SN bled	2-captured SP bled	3-captured not bled
not captured	1	0	0	0
SN captured	0	γ_{SN}	0	$1-\gamma_{SN}$
SP captured	0	0	γ_{SP}	$1 - \gamma_{SP}$

Where, conditioned on have being captured the current session, γ_{SN} and γ_{SP} refer respectively to the probability a seronegative and a seropositive have to be bled.

TRAP-DEPENDENCE PARAMETERIZATION (used for E1 data set)

When modeling trap-dependence (trap-happiness in our case) we followed the parameterization indicated by [42]. This parameterization consists of a modification of the number of states and an additional transition parameter step that specifically addresses trap-dependence. States are hence defined according to the fact one individual was captured just before (trap Aware) of the current session or not (trap Unaware). This way the capture probabilities appear as a step of transition parameters, more details are given in [42]. In our case we defined five underlying biological states:

† - Death
$SN_{(U)}$ - Seronegative alive not captured at the previous session
$SN_{(A)}$ - Seronegative alive captured at the previous session
$SP_{(U)}$ - Seropositive alive not captured at the previous session
$SP_{(A)}$ - Seropositive alive captured at the previous session

The events remained the same as before:
0 - Rabbit not captured
1 - Rabbit captured, bled and classified as seronegative
2 - Rabbit captured, bled and classified as seropositive
3 - Rabbit captured, not bled

INITIAL STATE

$SN_{(U)}$	$SN_{(A)}$	$SP_{(U)}$	$SP_{(A)}$
$1-\pi$	0	π	0

where π is the probability, at a certain occasion *t*, a first captured rabbit has to be seropositive. Note that: (*i*) a previously captured individual has, by definition, zero probability of being first-time captured at the current occasion and, (*ii*) no age or sex effect has been modeled on this parameter.

STATE TRANSITION

Step 1 Survival; this step computes the probability that an individual first captured on occasion *t* survives in the interval between *t* and $t + 1^-$ (the instant just before $t + 1$).

	$SN_{(U)}$	$SN_{(A)}$	$SP_{(U)}$	$SP_{(A)}$	†
$SN_{(U)}$	$\varphi_{SN(U)}$	0	0	0	$1-\varphi_{SN(U)}$
$SN_{(A)}$	0	$\varphi_{SN(A)}$	0	0	$1-\varphi_{SN(A)}$
$SP_{(U)}$	0	0	$\varphi_{SP(U)}$	0	$1-\varphi_{SP(U)}$
$SP_{(A)}$	0	0	0	$\varphi_{SP(A)}$	$1-\varphi_{SP(A)}$
†	0	0	0	0	1

Where φ_{SN} and φ_{SP} refer respectively to the probability a seronegative or a seropositive has of surviving in the interval after its first capture and notation (U) and (A) refers respectively to individuals not captured (trap-unaware) and captured (trap-aware) at the previous occasion. In E-SURGE, we constrained $\varphi_{SN(U)} = \varphi_{SN(A)}$ and $\varphi_{SP(U)} = \varphi_{SP(A)}$ so that the trap-awareness process had no effect on survival probabilities.

Step 2 Seroconversion (conditional on Survival); this step computes the probability a survived individual has of changing its state at $t + 1^-$ (the instant just before t + 1).

	$SN_{(U)}$	$SN_{(A)}$	$SP_{(U)}$	$SP_{(A)}$	†
$SN_{(U)}$	$1-\psi_{SN(U)}$	0	$\psi_{SN(U)}$	0	0
$SN_{(A)}$	0	$1-\psi_{SN(A)}$	0	$\psi_{SN(A)}$	0
$SP_{(U)}$	$\psi_{SP(U)}$	0	$1-\psi_{SP(U)}$	0	0
$SP_{(A)}$	0	$\psi_{SP(A)}$	0	$1-\psi_{SP(A)}$	0
†	0	0	0	0	1

Where, conditioned on having survived the current interval: (*i*) ψ_{SN} and ψ_{SP} refer respectively to the probability a seronegative has to become a seropositive and a seropositive has to become a seronegative and, (*ii*) notation "(U)" and "(A)" have the same meaning as from step 1. In E-SURGE, we have constrained $\psi_{SN(U)} = \psi_{SN(A)}$ and $\psi_{SP(U)} = \psi_{SP(A)}$.

Step 3 Trap-Dependence (conditional on Survival and Seroconversion); this step computes the probability an individual alive and in a certain serological state at $t + 1^-$ (the instant just before $t + 1$) has of changing its trap-awareness state between $t + 1^-$ and $t + 1^+$, i.e. between the instant just before the $t + 1$ and the instant just after it.

	$SN_{(U)}$	$SN_{(A)}$	$SP_{(U)}$	$SP_{(A)}$	†
$SN_{(U)}$	$1-\tau_{SN(U)}$	$\tau_{SN(U)}$	0	0	0
$SN_{(A)}$	$1-\tau_{SN(A)}$	$\tau_{SN(A)}$	0	0	0
$SP_{(U)}$	0	0	$1-\tau_{SP(U)}$	$\tau_{SP(U)}$	0
$SP_{(A)}$	0	0	$1-\tau_{SP(A)}$	$\tau_{SP(A)}$	0
†	0	0	0	0	1

Where, conditioned on having survived the current interval and being in a certain serological state in $t + 1^-$: (*i*) τ_{SN} and τ_{SP} refer respectively to the probability of being trap-aware at $t + 1^+$, i.e. of being captured at $t + 1$, and, (*ii*) notation "(U)" and "(A)" have the same meaning as from previous step 1.

EVENT

The Event probabilities here relates uniquely to the State Assignment process.

Step 1 State Assignment (conditional on Capture); this step computes, conditional on being captured at $t + 1$ (see step 3 in Transition), the probability one individual has to be bled when captured. In columns are the events as they are coded in the data set.

	0-not captured	1-captured SN bled	2-captured SP bled	3-captured not bled
$SN_{(U)}$	1	0	0	0
$SN_{(A)}$	0	γ_{SN}	0	$1-\gamma_{SN}$
$SP_{(U)}$	1	0	0	0
$SP_{(A)}$	0	0	γ_{SP}	$1 - \gamma_{SP}$
†	1	0	0	0

Where, conditioned on have being captured the current session, γ_{SN} and γ_{SP} refer respectively to the probability a seronegative and a seropositive have to be bled.

Additional file 3. Survival estimates with 95% CI for each enclosure (E1, E2 and E3). Survival estimates in the three enclosures as depending on the main effects found through model selection.

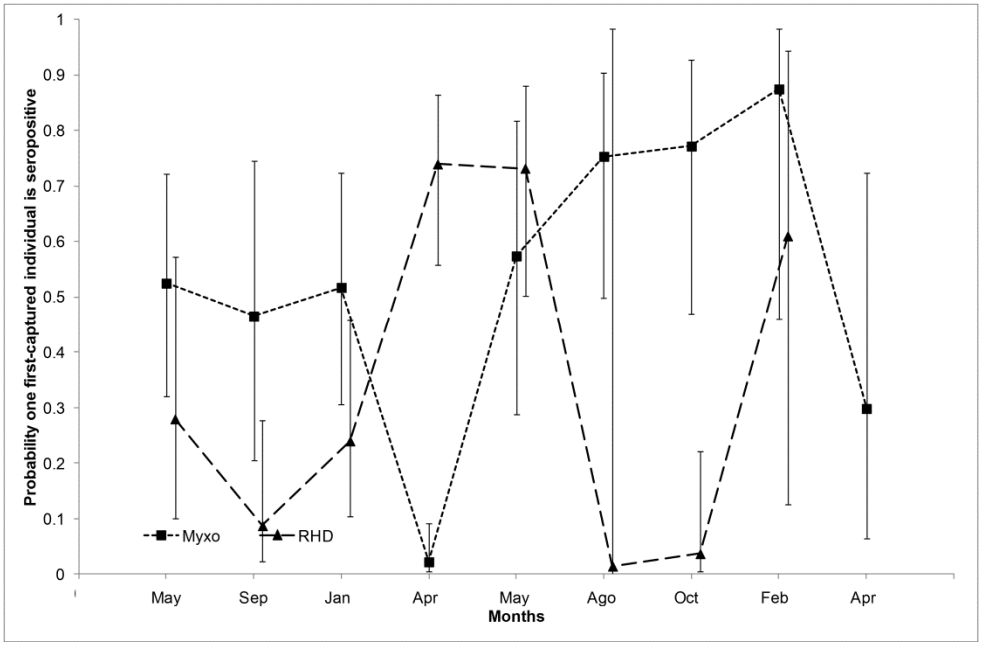
Main effects	Estimates	Low 95% CI	High 95% CI
E1 MYXO			
juv mal	0.84	0.79	0.88
juv fem	0.83	0.78	0.87
ad mal	0.91	0.89	0.92
ad fem	0.93	0.92	0.95
E1 RHD			
juv mal SN	0.92	0.83	0.97
juv mal SP	0.96	0.47	1
juv fem SN	0.88	0.80	0.94
juv fem SP	0.87	0.71	0.95
ad mal SN	0.95	0.92	0.97
ad mal SP	0.83	0.77	0.88
ad fem SN	0.95	0.93	0.96
ad fem SP	0.92	0.88	0.95
E2 MYXO			
juv mal SN	0.94	0.80	0.99
juv mal SP	0.78	0.61	0.89
juv fem SN	0.98	0.51	1
juv fem SP	0.65	0.47	0.80
ad mal SN	0.89	0.84	0.92
ad mal SP	0.95	0.87	0.98
ad fem SN	0.96	0.91	0.99
ad fem SP	0.87	0.82	0.91
E2 RHD			
juv SN	0.92	0.86	0.95
juv SP	0.74	0.59	0.84
ad SN	0.92	0.89	0.94
ad SP	0.94	0.90	0.97
E3 MYXO			
SN	0.86	0.83	0.89
SP	0.94	0.92	0.96
E3 RHD			
juv SN	0.88	0.82	0.92
juv SP	0.81	0.68	0.90
ad SN	0.92	0.89	0.95
ad SP	0.96	0.83	0.99

Notation: juv, Juveniles; ad, Adults; fem, Females; mal, Males; SN, Seronegatives; SP, Seropositives.

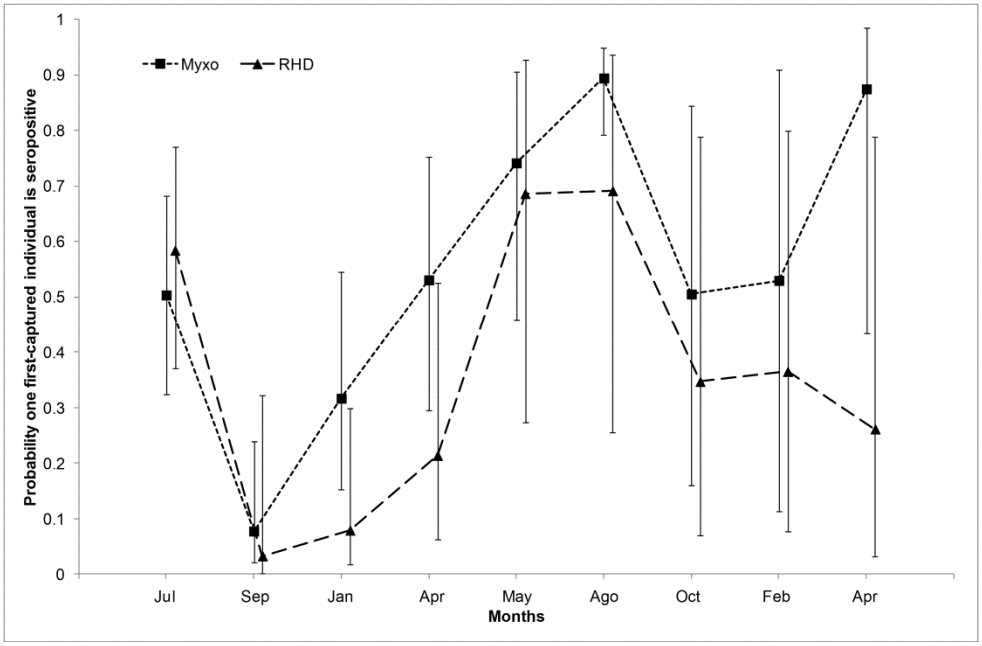
Additional file 4. Seroconversion estimates with 95% CI for each enclosure (E1, E2 and E3). Seroconversion estimates in the three enclosures as depending on the main effects found through model selection. No age effect was tested because juveniles remain juveniles only for a short time and no recapture from one individual in juvenile state exists.

Main effects	**Estimates**	**Low 95% CI**	**High 95% CI**
E1 MYXO			
mal SN SP	0.39	0.27	0.54
mal SP SN	0.07	0.03	0.17
fem SN SP	0.22	0.15	0.32
fem SP SN	0	0	0
E1 RHD			
SN SP	0.28	0.23	0.35
SP SN	0.13	0.09	0.18
E2 MYXO			
mal SN SP	0.04	0.01	0.24
mal SP SN	0.08	0.01	0.42
fem SN SP	0.31	0.18	0.47
fem SP SN	0.03	0.01	0.14
E2 RHD			
mal SN SP	0.14	0.07	0.29
mal SP SN	0.15	0.04	0.41
fem SN SP	0.44	0.28	0.61
fem SP SN	0.18	0.08	0.34
E3 MYXO			
SN SP	0.20	0.12	0.30
SP SN	0	0	0
E3 RHD			
mal SN SP	0.13	0.10	0.18
mal SP SN	0.68	0.54	0.79
fem SN SP	0.14	0.09	0.20
fem SP SN	0.73	0.62	0.82

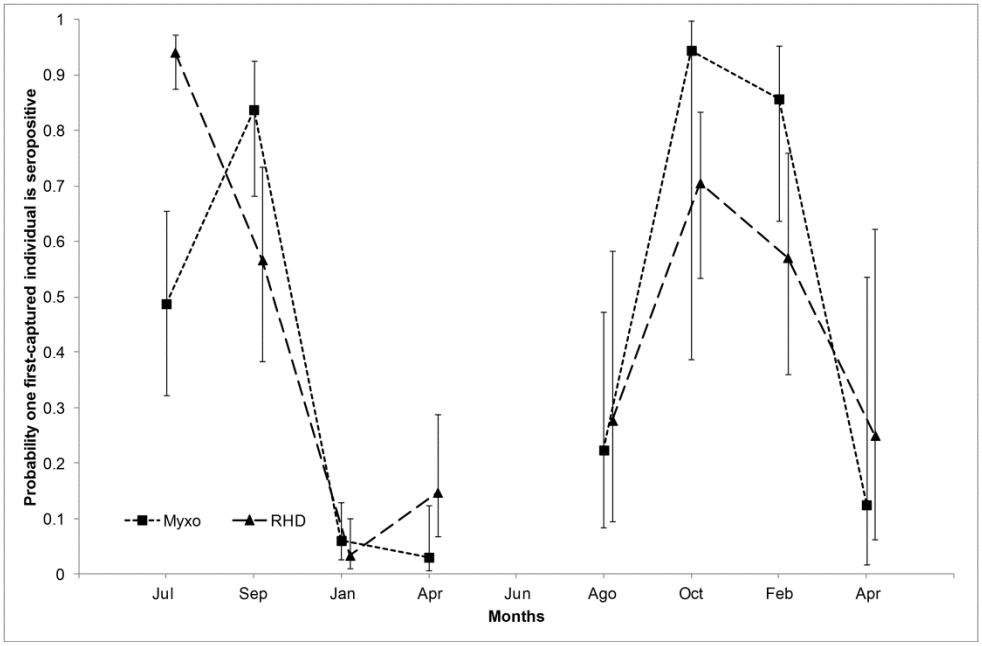
Notation: as from Additional file 3.



Additional file 5. Myxoma virus and RHDV antibody prevalence in enclosure E1. Myxoma virus and rabbit haemorrhagic disease virus seroprevalences over the study period as proxied by Initial State in enclosure E1. Prevalence estimates refer to the probability one first captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represents 95% confidence intervals.



Additional file 6. Myxoma virus and RHDV antibody prevalence in enclosure E2. Myxoma virus and rabbit haemorrhagic disease virus seroprevalences over the study period as proxied by Initial State in enclosure E2. Prevalence estimates refer to the probability one first-captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represent 95% confidence intervals



Additional file 7. Myxoma virus and RHDV antibody prevalence in enclosure E3. Myxoma virus and Rabbit Hemorrhagic Disease Virus seroprevalences over the study period as proxied by Initial State in enclosure E3. Prevalence estimates refer to the probability one first-captured individual is seropositive. This probability is corrected for the specific session probability of capture of seronegatives and seropositives. Vertical bars represent 95% confidence intervals.

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COCCIDIAN AND NEMATODE INFECTIONS INFLUENCE PREVALENCE OF ANTIBODY TO MYXOMA AND RABBIT HEMORRHAGIC DISEASE VIRUSES IN EUROPEAN RABBITS

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ABSTRACT: The interaction among several parasites in European rabbits (*Oryctolagus cuniculus*) is crucial to host fitness and to the epidemiology of myxomatosis and rabbit hemorrhagic disease. These diseases have caused significant reductions in rabbit populations on the Iberian Peninsula. Most studies have focused on the epidemiology and pathogenesis of these viruses individually, and little is known about interactions between these viruses and other parasites. Taking advantage of an experimental restocking program in Spain, the effects of coccidian and nematode infections on the probability of having detectable antibody to myxoma and rabbit hemorrhagic disease viruses were tested in European wild rabbits. For 14 mo, we monitored rabbit abundance and parasite loads (coccidia and nematodes) in three reintroduced rabbit populations. While coccidian and nematode loads explained seasonal antibody prevalences to myxoma virus, the pattern was less clear for rabbit hemorrhagic disease. Contrary to expectations, prevalence of antibody to myxoma virus was inversely proportional to coccidian load, while nematode load seemed to play a minor role. These results have implications for viral disease epidemiology and for disease management intended to increase rabbit populations in areas where they are important for ecosystem conservation.

Key words: Coinfection, disease management, immune response, *Oryctolagus cuniculus*, restocking, viral diseases.

INTRODUCTION

The arrival of two viral diseases, myxomatosis in the 1950s and rabbit hemorrhagic disease (RHD) in the 1980s, was the primary cause of a significant reduction in the European rabbit (*Oryctolagus cuniculus*) population on the Iberian Peninsula (Delibes-Mateos et al., 2009). Because the European rabbit is the staple prey for more than 30 predators in Spain, this population crash caused a significant perturbation to the ecosystem (Delibes-Mateos et al., 2008). Populations of some predators, including the Iberian lynx (*Lynx pardina*) and the Iberian Imperial Eagle (*Aquila adalberti*), are now endangered (Ferrer and Negro, 2004); therefore, the recovery of wild rabbit populations has become a major goal of nature conservation in Spain. Although management techniques have been applied (Moreno and Villafuerte, 1995; Calvete et al., 2004; Cabezas and Moreno, 2007; Rouco et al., 2008, 2011), they have been only partly successful in mitigating viral diseases, and annual outbreaks of myxomatosis and RHD cause high mortality in European Mediterranean ecosystems. Most studies have focused on the epidemiology and pathogenesis of these diseases separately (Calvete et al., 2002), and the potential role of coinfection has been so far neglected.

Studies have shown different coinfection relationships between myxoma virus (MV) and parasite infections (Boag, 1988; Boag et al., 2001; Lello et al., 2005). Two long-term monitoring studies (Cattadori et al., 2007, 2008) have led to two important conclusions concerning the dynamics of coinfection under natural conditions. Rabbits infected with MV are more susceptible to nematode infection, and rabbits with existing nematode infestations suffer longer MV infections.

The immune response plays a key role in these interactions. It is generally accepted that after infection, naïve T helper (Th) cells begin to differentiate into Th1 and Th2 cells, each characterized by a specific type of interleukin (IL). Thus, the response triggered by MV (as a microparasite) biases the system toward Th1, but immune defense against nematodes (macroparasites) requires a Th2 response (Cox, 2001; Pedersen and Fenton, 2006). An experimental study showed that if both pathways occur simultaneously, the relaxation of the immune response provokes higher mortality (Kerr et al., 2004). Thus, the interactions between the two viral diseases and other parasites remain unclear, and there is little information about the ways in which other factors, such as host age, sex, body condition, reproductive stage, season, or abundance, influence these pathogen interactions.

We tested whether the loads of microparasites (belonging to the family *Eimeriidae*, subclass Coccidiasina) and macroparasites (gastrointestinal nematodes) influence the ability of rabbits to generate an appropriate immunologic response against MV and rabbit hemorrhagic disease virus (RHDV). Because susceptibility to a given pathogen would be affected by the ongoing cytokine response due to a preexisting infection (Graham et al., 2007), we tested two predictions: (1) Populations with higher nematode loads will present lower antibody prevalences to both viruses, because gastrointestinal nematodes would polarize the immune system toward Th2, and (2) populations with higher coccidian loads will have higher antibody prevalences, because coccidia and viruses are regulated by the same Th1 pathway (Yun et al., 2000; Pedersen and Fenton, 2006).

MATERIALS AND METHODS

Study area

The study was carried out in a mountainous area in SW Spain (37°49′N, 05°15′W), between 100 and 700 m elevation. Climate is Mediterranean with hot, dry summers and cool, wet winters (Pinilla et al., 1995). The rabbit restocking program was carried out in 2008 in the Hornachuelos Natural Park for conservation purposes. Rabbits for restocking were captured in a natural area 50 km away, within the range of the same subspecies (*Oryctolagus cuniculus algyrus*). The objective was to create three areas with high rabbit population density, where animals would maximize their productivity and become donors for neighboring areas. Three plots (4 ha each) were surrounded by 2.5-m-high chain-link fence and were provided with shelter and ad-libitum food and water. One release (150 rabbits) was made into each netting fence in May 2008. We took advantage of this existing conservation program for our study.

Monitoring of rabbit populations

From January 2009 to April 2010, five live-trapping sessions were conducted in each plot. Most captured animals (see following) were handled at the capture site and immediately released. For all specimens, we recorded sex, weight to 0.1 g, and ear and tarsus length (measured with a caliper to 0.01 mm). Age was estimated according to body weight (rabbits with >800 g were considered adults [Moreno et al., 2004]), and each rabbit was marked with a numbered ear tag. Among 6,605 captured rabbits, 563 adults (253 females and 310 males) without clinical signs of myxomatosis or RHD were selected and transported to a field laboratory within each plot. Blood samples (1–2.5 mL) were taken from each animal via the ear vein for determination of antibody status. In the end, all rabbits were released in their own warren. None of the sampled rabbits had been previously captured, so no animals were sampled twice.

Serology

Blood samples were centrifuged in Eppendorff tubes, and serum was kept at −80 C until analyzed. Serum antibody concentrations (see following) against MV and RHDV were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (described in the following) according to diagnostic techniques recommended by the World Organisation for Animal Health (OIE, 2012), in the Physiological Ecology Laboratory of the Doñana Biological Station, Seville, Spain.

For MV, sera were diluted 1:40, and a relative immunity index (RI) was obtained as a coefficient between the optical density (OD) of controls (positive and negative) and the OD of the sample. The RIs ranged from 1 to 10. All rabbits with RI>2 were considered

antibody-positive (CIVTest cuni, Hipra Laboratory, Gerona, Spain). Tests for antibody against RHDV were carried out using commercial ELISA *Ingezim rabbit* (Ingenasa Laboratory, Madrid, Spain). Sera were screened at 1:200, 1:400, 1:800, and 1:1,600, and samples with an OD>0.3 were considered positive. Specificity and sensitivity of this test were 83.1% and 98.5%, respectively, and had a 93% correspondence with the reference technique (OIE, 2012).

Rabbit abundance

Rabbit abundance was estimated using pellet counts in fixed recounting stations (0.5 m^2 each) located inside the fences, in the same habitat type (Cabezas and Moreno, 2007). Thirty fixed stations per plot were randomly arranged. Monthly, pellets were counted and removed from each counting station. Because persistence of pellets in the field may vary according to habitat and season (Iborra and Lumaret, 1997; Palomares 2001), the daily persistence of pellets was calculated in our study area following Palomares (2001).

Parasitologic analysis

To analyze the load of coccidia and nematodes within each plot, 20 samples with five fresh pellets each were randomly collected each month (Coudert et al., 2000). To avoid samples from juveniles, only pellets >6 mm in diameter were collected (Rouco et al., 2012). Fecal egg and oocyst counts were determined by the modified McMaster technique (Raynaud, 1970), and results are expressed as oocysts per gram (opc) of feces for coccidia or eggs per gram (epg) of feces for nematodes. No other group of parasites was found.

Data analyses

To evaluate the predictions, a model selection approach was followed performing generalized linear mixed models (GLMMs) using a binomial distributed error with a logistic link function. The probability of being antibody-positive (categorical variable: antibody-negative=0, antibody-positive=1) for both MV and RHDV (as response variables) was explained by the single effect and the two-way interactions of the following explanatory variables: nematode load (epg), coccidian load (opg), rabbit abundance (pellets/m^2), sex, month, and body condition. Body condition was obtained with residual values after performing a simple linear regression between log-transformed body weight and tarsus length (Cabezas et al., 2006). Because animals were systematically sampled from the same plots, the study site was included as a random factor in our analyses. Because all samples came from adult animals, age was excluded from the analysis.

Model selection was performed following a theoretic information approach based on Akaike's information criterion (AIC; Anderson et al., 2001; Johnson and Omland, 2004). Briefly, for each candidate model, the AIC was estimated by selecting the model with the lowest value; we then ranked the remaining competing models according to their AIC value and subsequently estimated their Akaike differences (Δi) with respect to the best model (lowest AIC). Subsequently, the Akaike weights (wi) were estimated, defined as the relative probability of each model to be the best one among those being compared (Anderson et al., 2001). The absence of a pattern in residual values of the selected models was confirmed (Zuur et al., 2009). In order to discuss the effect size, the explained deviance of the best model and the pure deviance of each variable were calculated (Zuur et al., 2007). Statistical analyses were done using R version 2.12.2. Specifically, GLMMs were implemented using the "lme4" package, R package version 0.999375-35 (R Development Core Team, University of Auckland, Auckland, New Zealand).

RESULTS

Myxoma virus antibody prevalence peaked seasonally in February and October and was similar in the three plots (Fig. 1). For RHDV, plots 2 and 3 (but not plot 1) had annual maxima in April and October (Fig. 1). Coccidian and nematode loads had specific monthly patterns with maximum values in April (Fig. 1).

The model selection showed that prevalence of antibody against MV was influenced by both coccidian and nematode loads in each plot (Table 1 and Fig. 2). In the best model for MV ($wi_{\text{Mo+Coccidian load+Nematode load}}$, Table 1), 16% of the observed variability in the probability of being antibody-positive was explained by month ($\beta_{Mo}=-0.04$, standard error [SE]=0.008, t-value=−5.4) and also for the coccidian ($\beta_{Coccidian}=-0.49$, $SE=0.05$, $t=-9.23$) and nematode ($\beta_{Nematodes}=-0.22$, $SE=0.03$, $t=-6.22$) loads in each plot. However, coccidian and nematode loads shared 1.7% of the explained deviance (they were positively correlated, $\beta=0.25$, $SE=0.03$, $t=6.8$, $R^2=7.7\%$). Correcting for

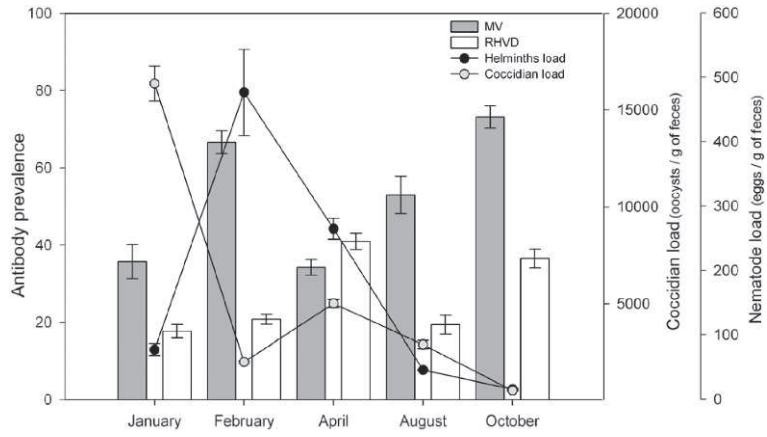

FIGURE 1. Average monthly prevalence of myxoma virus (MV) and rabbit hemorrhagic disease virus (RHDV) and coccidian and nematode (helminth) load in three European rabbit (*Oryctolagus cuniculus*) populations.

this, the pure effect of coccidian load was about 4%, and that of nematode load was 1.4%. The influence of parasites persisted after correcting for season, being less intense in winter-spring (e.g., January, February, and April, $\beta_{Coccidian} = -0.37$, $SE = 0.06$, $t = -5.7$, and $\beta_{Nematodes} = -0.94$, $SE = 0.18$, $t = -5.2$, explaining for both factors 14% of the observed variability in MV antibody prevalence) than in summer-autumn (e.g., August and October, $\beta_{Coccidian} = -0.48$, $SE = 0.07$, $t = -6.5$, and $\beta_{Nematodes} = -0.36$, $SE = 0.14$, $t = -2.46$, explaining for both factors 15.8% of the

TABLE 1. Model selection for probability of having detectable antibody to myxoma and rabbit hemorrhagic disease (RHD) viruses in a sample of 563 European wild rabbits (*Oryctolagus cuniculus*) in the southwestern Iberian Peninsula. Models with substantial support for being the best model are represented in bold.[a]

Biological models	*K*	AIC	Δ*i*	*wi*
Myxoma virus				
Mo+coccidian load+nematode load	6	707.39	0	0.99
Coccidian load+nematode load	5	726.24	18.80	<0.001
Mo	3	810.98	103.48	<0.001
RHD virus				
Abundance+coccidian load+nematode load	6	706.97	0	0.30
Mo	3	707.06	0.09	0.29
Helminth load	4	708.65	1.68	0.13
Coccidian load+nematode load	5	709.54	2.57	0.09
Body condition+nematode load	5	710.62	3.65	0.05
Body condition	4	711.03	4.05	0.04
Abundance+coccidian load	5	712.48	5.51	0.02
Abundance+nematode load	5	713.23	6.26	0.01
Abundance	4	713.25	6.28	0.01
Mo+nematode load	5	713.33	6.36	0.01
Coccidian load	4	713.38	6.41	0.01
Sex	4	714.21	7.24	<0.001
Body condition+coccidian load	5	715.73	8.76	<0.001

[a] *K* = number of parameters including intercept; AIC = Akaike Information Criterion; Δ*i* = difference of AIC with respect to the best model; *wi* = Akaike weight; Mo = null model only with the constant term.

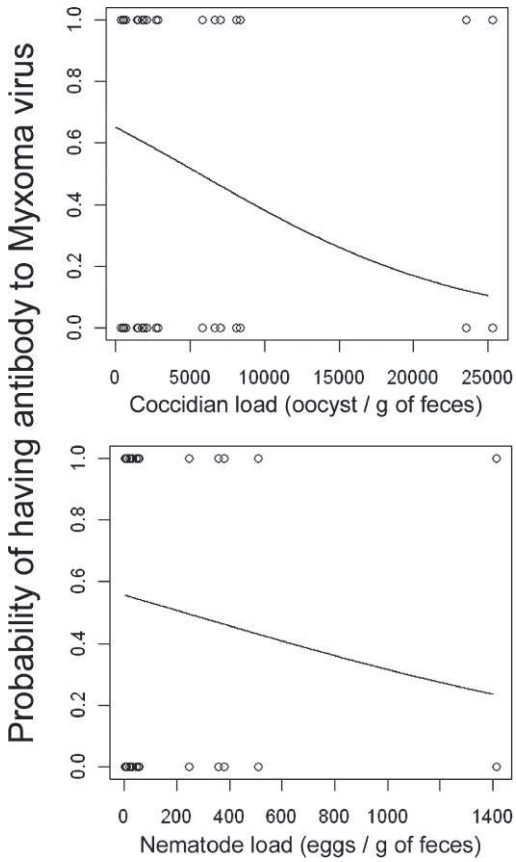

FIGURE 2. Effect of coccidian and nematode load on probability of having detectable antibody to myxoma virus in three European rabbit (*Oryctolagus cuniculus*) populations.

observed variability in MV antibody prevalence).

Other factors, such as strict rabbit abundance ($\beta_{Abundance}$=0.15, SE=0.05, t=3.05, explaining 1.1% of the observed variability in MV antibody prevalence) or body condition ($\beta_{Body\ condition}$=0.55, SE=0.34, t=1.5, explained deviance=0.2%), had a slight positive effect on the probability of being antibody-positive. Sex had no effect on the rate of MV antibody prevalence (β_{Sex}=0.04, SE=0.04, t=0.9, deviance explained=0.07%). There was no clear effect of parasite load, rabbit abundance, body condition, or sex for RHDV antibody-prevalence, since the null model (Mo) was placed at 0.09 points from the best model, and the AIC weights for both models were similar (Table 1).

DISCUSSION

Parasite load was a clear explanatory factor for prevalence of antibody to MV but not RHDV in European hares. However, contrary to our initial prediction, in the three populations, higher prevalence to MV occurred at low coccidian load. Before discussing our results, a methodological limitation should be considered. Data on antibody prevalence come from individual rabbits, while data on coccidian and nematode load represent the entire population. It was impossible to obtain both blood and fresh pellet samples from the same captured rabbits, as would have been ideal. However, both kinds of samples were taken simultaneously and always represent the same adult population. Thus, our results are likely to be a reliable approximation that could be tested in the laboratory.

Coccidian infection is one of the main predisposing factors for intestinal enteropathies and can cause higher fatality rates than nematodes (Varga, 1982; Hobbs et al., 1999), especially as animals that have recovered from coccidiosis are immunocompromised (Yun et al., 2000). Nevertheless, in our study, coccidian infections were more common than nematode infections in the wild, as others have also found (Peeters et al., 1981). Thus, it is likely that coccidian load has played a key role in generating an immunologic response, since there is higher probability of both pathogens (MV and coccidian) occurring in the same animal. Contrary to our initial hypothesis, populations with lower coccidian load had higher prevalences of antibody to MV. These patterns of coinfection (e.g., nematode-coccidian-MV) could be partially due to an immunosuppressive effect of MV by decreasing circulating Th cells, and the remaining Th cells polarize the system to Th2 (Jeklova et al., 2008). Thus, the immune response against nematodes is

not completely disrupted by the response to MV (Cattadori et al., 2008), but dealing with coccidia and MV simultaneously requires a high Th1 response that the immune system is unable to produce. In spite of this, the trade-off of the Th1/Th2 response must not be the only mechanism of the immune system to deal with coinfections (Cox, 2001). Other ecologic and environmental factors likely play an important role that was not considered in this study. In any case, it seems to be clear that coccidian control could play a key role in combating rabbit diseases (Peeters et al., 1984).

In the context of RHDV coinfection, our model showed no relationship between the explanatory variables and the ability to develop RHDV antibody. Differences in the pathogenesis of both viruses could explain these results. The way our explanatory variables work is easier to understand in an immunosuppressive virus (MV), and, therefore, different variables should be considered to understand the ability to mount an antibody response to RHDV.

We were surprised that the model did not select rabbit abundance as an influencing variable because several authors have reported a strong relationship between this variable and MV antibody prevalence (Calvete et al., 2002; Fouchet et al., 2008). Moreover, the transmission of MV is density dependent; the virus disappears after an epidemic in smaller populations but becomes endemic in large ones (Fouchet et al., 2008). Because antibodies against MV are maintained in rabbits for life (Fouchet et al., 2008), and the virus is widespread in the wild in southern Spain (Garcıa-Bocanegra et al., 2010), MV antibody prevalence is higher in dense populations. The absence in our analysis of data from juvenile rabbits, responsible for the overall seroconversion rates, was probably the cause of these results.

Our results agree with those of Blasco et al. (1996), who found that the highest prevalences of parasitization occurred during the breeding season, preceding peak population abundance; thus, a delay between coccidian and nematode levels and rabbit abundance occurs. For this reason, the highest MV seroconversion rates would occur in rabbit populations that have lower parasite loads and higher densities.

Several authors have demonstrated that rabbit abundance plays a similar role in the epizootiology of RHDV and MV (Calvete et al., 2002; Cooke, 2002). Yet, we did not find a clear relationship between population abundance and RHDV antibody prevalence. Further studies are needed to understand this absence of a relationship.

Although more information is required, antibody prevalence to MV clearly depended heavily on coccidian load. High prevalence occurred when coccidian load was low, while nematode load played a minor role in this process. Our results have implications not only for the viral disease epizootiology, but ultimately, for disease management aimed to increase rabbit populations in areas where the rabbit is a keystone species for ecosystem conservation.

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LITERATURE CITED

Anderson DR, Burham KP, White GA. 2001. Kullback-Leibler information in resolving natural

resource conflicts when definitive data exist. *Wildl Soc Bull* 29:1260–1270.

Blasco S, Torres J, Feliu C, Casanova JC, Miquel J, Moreno S. 1996. The helminth fauna of *Oryctolagus cuniculus* (Linnaeus, 1758) in the Iberian Peninsula. Faunisitic and ecological considerations. *Parasite* 4:327–333.

Boag B. 1988. Observations on the seasonal incidence of myxomatosis and its interactions with helminth parasites in the European rabbit (*Oryctolagus cuniculus*). *J Wildl Dis* 24:450–455.

Boag B, Lello J, Fenton A, Tompkins DM, Hudson PJ. 2001. Patterns of parasite aggregation in the wild European rabbit (*Oryctolagus cuniculus*). *Int J Parasit* 31:1421–1428.

Cabezas S, Moreno S. 2007. An experimental study of translocation success and habitat improvement in wild rabbits. *Anim Conserv* 10:340–348.

Cabezas S, Calvete C, Moreno S. 2006. Vaccination success and body condition in the European wild rabbit: Applications for conservation strategies. *J Wildl Manag* 70:1125–1131.

Calvete C, Estrada R, Lucientes J, Osacar JJ, Villafuerte R. 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. *Vet Record* 150:776–782.

Calvete C, Estrada R, Osacar JJ, Lucientes J, Villafuerte R. 2004. Short-term negative effects of vaccination campaigns against myxomatosis and viral hemorrhagic disease (VHD) on the survival of European wild rabbits. *J Wildl Manag* 68:198–205.

Cattadori IM, Albert R, Boag B. 2007. Variation in host susceptibility and infectiousness generated by co-infection: The myxoma-*Trichostrongylus retortaeformis* case in wild rabbits. *J Roy Soc Interface* 4:831–840.

Cattadori IM, Boag B, Hudson PJ. 2008. Parasite co-infection and interaction as drivers of host heterogeneity. *Int J Parasitol* 38:371–380.

Cooke BD. 2002. Rabbit haemorrhagic disease: Field epidemiology and management of wild rabbit populations. *Rev Sci Off Int Epizoot* 21:347–358.

Coudert P, Licois D, Drouet-viard F, Provôt F. 2000. Coccidiosis. *In Enfermedades del conejo*. Tomo II. Mundi-Prensa, Madrid, Spain, pp. 219–234.

Cox FEG. 2001. Concomitant infections, parasites and immune responses. *Parasitology* 122:23–28.

Delibes-Mateos M, Delibes M, Ferreras P, Villafuerte R. 2008. Key role of European rabbits in the conservation of the western Mediterranean basin hotspot. *Conserv Biol* 22:1106–1117.

Delibes-Mateos M, Ferreras P, Villafuerte R. 2009. European rabbit population trends and associated factors: A review of the situation in the Iberian Peninsula. *Mamm Rev* 39:124–140.

Ferrer M, Negro JJ. 2004. The near extinction of two large European predators: Super specialists pay a price. *Conserv Biol* 18:344–349.

Fouchet D, Guitton JS, Marchandeau, Pontier D. 2008. Impact of myxomatosis in relation to local persistence in wild rabbit populations: The role of waning immunity and the reproductive period. *J Theor Biol* 250:593–605.

Garcia-Bocanegra I, Astorga RJ, Napp S, Casal J, Huerta B, Borge C, Arenas A. 2010. Myxomatosis in wild rabbit: Design of control programs in Mediterranean ecosystems. *Prev Vet Med* 93:42–50.

Graham AL, Cattadori IM, Liyd-Smith JO, Ferrari MJ, Bjørnstad O. 2007. Transmission consequences of coinfection: Cytokines writ large? *Trends Parasitol* 23:284–291.

Hobbs RP, Twigg LE, Elliot AD, Wheeler AG. 1999. Evaluation of the association of parasitism with mortality of wild European rabbits *Oryctolagus cuniculus* (L) in south Western Australia. *J Parasitol* 85:803–808.

Iborra O, Lumaret JP. 1997. Validity limits of the pellet group counts in wild rabbit (*Oryctolagus cuniculus*). *Mammalia* 61:205–218.

Jeklova E, Levaa L, Matiasovica J, Kovarcika K, Kudlackovaa H, Nevorankovaa Z, Psikala I, Faldyna M. 2008. Characterisation of immunosuppression in rabbits after infection with myxoma virus. *Vet Microbiol* 129:117–130.

Johnson JB, Omland KS. 2004. Model selection in ecology and evolution. *Trends Ecol Evol* 19:101–108.

Kerr PJ, Perkins HD, Inglis B, Stagg R, McLaughlin E, Collins SV, van Leeuwen BH. 2004. Expression of rabbit IL-4 by recombinant myxoma viruses enhances virulence and overcomes genetic resistance to myxomatosis. *Virology* 324:117–128.

Lello J, Boag B, Hudson PJ. 2005. The effect of single and concomitant pathogen infections on condition and fecundity of the wild rabbit (*Oryctolagus cuniculus*). *Int J Parasitol* 35:1509–1515.

Moreno S, Villafuerte R. 1995. Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain. *Biol Conserv* 73:81–85.

Moreno S, Villafuerte R, Cabezas S, Lombardi L. 2004. Wild rabbit restocking for predator conservation in Spain. *Biol Conserv* 118:183–193.

OIE (World *Organisation for* Animal *Health*). 2012. *Technical disease cards*. http://www.oie.int/en/our-scientific-expertise/specific-information-and-recommendations/technical-disease-cards/. Accessed September 2012.

Palomares F. 2001. Comparison of 3 methods to estimate rabbit abundance in Mediterranean environment. *Wildl Soc Bull* 29:578–585.

Pedersen A, Fenton A. 2006. Emphasizing the ecology in parasite community ecology. *Trends Ecol Evol* 22:133–139.

Peeters JE, Geeroms R, Froyman R, Halen P. 1981. Coccidiosis in rabbits: A field study. *Res Vet Sci* 30:328–334.

Peeters JE, Pohl P, Gharlier G. 1984. Infectious agents associated with diarrhoea in commercial rabbits: A field study. *Ann Rech Vétér* 15:335–340.

Pinilla R, Tamajón R, Muñoz J. 1995. Vegetación actual. *In Reconocimiento biofísico de espacios naturales protegidos. Parque Natural Sierra de Hornachuelos. Consejería de Medio Ambiente*. Junta de Andalucía, Andalucía, Spain, pp. 207–247. http://www.juntadeandalucia.es/medioambiente/web/Bloques_Tematicos/Publicaciones_Divulgacion_Y_Noticias/Documentos_Tecnicos/Reconocimiento_Hornachuelos/PDFs/207_247.pdf. Accessed September 2012.

Raynaud JP. 1970. Etude de l'efficacité d'une méthode de coproscopie quantitative pour le diagnostic de routine et le contrôle des infestations parasitaires des bovins, ovins, équins et porcins. *Ann Parasitol Hum Comp* 45:321–342.

Rouco C, Ferreras P, Castro F, Villafuerte R. 2008. The effect of exclusion of terrestrial predators on short-term survival of translocated European wild rabbits. *Wildl Res* 35:625–632.

Rouco C, Villafuerte R, Castro F, Ferreras P. 2011. Effect of artificial warren size on a restocked European wild rabbit population. *Anim Conserv* 14:117–123.

Rouco C, Starkloff A, Delibes-Mateos M, Schubert M, Rödel HG. 2012. Differentiation of animals from different age classes by means of pellet size: Assessment of a field method in European rabbits. *Mamm Biol* 77:451–454.

Varga I. 1982. Large-scale management systems and parasite populations: Coccidia in rabbits. *Vet Parasitol* 11:69–84.

Yun CH, Lillehoj HS, Lillehoj EP. 2000. Intestinal immune responses to coccidiosis. *Devel Comp Immun* 24:303–324.

Zuur AF, Ieno EN, Smith GM. 2007. *Analysing ecological data*. Springer, New York, New York, USA, 672 pp.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. *Mixed effects models and extension in ecology with R*. Springer, New York, New York, USA, 574 pp.

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Effects of myxoma virus and rabbit hemorrhagic disease virus on the physiological condition of wild European rabbits: Is blood biochemistry a useful monitoring tool?

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ABSTRACT

Myxomatosis and rabbit hemorrhagic disease (RHD) are the major viral diseases that affect the wild European rabbit (*Oryctolagus cuniculus*). These diseases arrived in Europe within the last decades and have caused wild rabbit populations to decline dramatically. Both viruses are currently considered to be endemic in the Iberian Peninsula; periodic outbreaks that strongly impact wild populations regularly occur. Myxoma virus (MV) and rabbit hemorrhagic disease virus (RHDV) alter the physiology of infected rabbits, resulting in physical deterioration. Consequently, the persistence and viability of natural populations are affected. The main goal of our study was to determine if blood biochemistry is correlated with serostatus in wild European rabbits. We carried out seven live-trapping sessions in three wild rabbit populations over a two-year period. Blood samples were collected to measure anti-MV and anti-RHDV antibody concentrations and to measure biochemical parameters related to organ function, protein metabolism, and nutritional status. Overall, we found no significant relationships between rabbit serostatus and biochemistry. Our main result was that rabbits that were seropositive for both MV and RHDV had low gamma glutamyltransferase concentrations. Given the robustness of our analyses, the lack of significant relationships may indicate that the biochemical parameters measured are poor proxies for serostatus. Another explanation is that wild rabbits might be producing attenuated physiological responses to these viruses because the latter are now enzootic in the study area.

1. Introduction

Diseases can represent major threats for wild animal populations because they can lead to decline and extinction (Viggers et al., 1993; Woodroffe, 1999; Morner et al., 2002). In fact, acquiring a better understanding of diseases and pathogens is a crucial but challenging task in wildlife conservation efforts (Deem et al., 2001). In ecosystems, host-pathogen relationships help shape patterns of species distribution and persistence (Dobson and Hudson, 1986; Thomas et al., 2005; Collinge and Ray, 2006; Hudson et al., 2006). Even though most previous studies have focused on one-host, one-pathogen systems, such dynamics are actually rare in nature. Individual hosts are often co-infected by multiple pathogens, which interact in complex ways with each other (Pedersen and Fenton, 2007). Therefore, studying the mechanisms underlying these interactions is of primary importance if we wish to predict how pathogens will affect host physiology and if we want to effectively control target and non-target parasite species.

Despite its relevance for wildlife conservation and management, the physiology of wild species is rarely studied because physiological parameters are difficult to quantify. Furthermore, it is challenging to combine physiological information with other data, such as antibody concentrations, at the population level. By incorporating indices of host physiological condition into population surveillance and monitoring efforts, we will gain deeper insight into the range of host responses and pathogen effects. Such tools could reveal the status of major pathogens within wild animal populations and provide a snapshot of a given animal's physiological state; consequently, they would serve as more straightforward means of assessing population condition. In this study, we used the wild European rabbit (*Oryctolagus cuniculus*) and its two main viral diseases, myxomatosis and rabbit hemorrhagic disease (RHD), as a model system.

At present, myxomatosis and RHD are endemic diseases in the Iberian Peninsula; they cause periodic outbreaks that significantly impact natural populations (Calvete et al., 2002). Outbreak patterns suggest

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that these viruses are in continuous recirculation and are largely associated with the breeding season; myxomatosis outbreaks occur predominantly in summer and autumn, while RHD outbreaks occur in winter and spring. It also appears that the viruses remain in the same areas from one year to the next (Calvete et al., 2002). Factors such as breeding season length and timing, host population size, vector abundance, and environmental conditions have major effects on the duration and potential impact of the epizootics and, ultimately, on virus persistence within populations (Fouchet et al., 2008). We currently have a good grasp of the epidemiology and pathology of myxomatosis and RHD, topics that are discussed extensively in the literature (e.g., Fenner and Woodroofe, 1953; Liu et al., 1984; Xu, 1991; Cooke, 2002; Calvete et al., 2002; Stanford et al., 2007; Abrantes et al., 2012; Santoro et al., 2014). Myxoma virus (MV) and rabbit hemorrhagic disease virus (RHDV) dramatically alter the physiology of infected rabbits. These alterations result in the deterioration of physical health, which we will hereafter refer to as physiological condition (Kerr and Donnelly, 2013). In general, an individual's physiological condition is negatively correlated with the degree of infection burden but positively correlated with immune function (Chandra and Newberne, 1977; Gershwin et al., 1985; Møller et al., 1998). Therefore, rabbits in poor physiological condition may also be more likely to become infected (Nelson and Demas, 1996; Tompkins and Begon, 1999; Beldomenico et al., 2008).

There is a need for straightforward, reliable methods for assessing the physiological condition of wild rabbits; past studies suggest that blood biochemistry could be helpful in this regard (Franzmann and Schwartz, 1988; Hellgren et al., 1989; Schroeder, 1987; Hellgren et al., 1993; Milner et al., 2003). Moreover, as compared to more conventional measures, biochemical parameters are highly sensitive, meaning they change to reflect an individual's physiological state in a matter of minutes. Consequently, the use of blood biochemistry may make it possible to identify rabbits experiencing extreme stress in general (Milner et al., 2003).

In this study we monitored blood chemistry and MV and RHDV serostatus in wild populations of the European rabbits. Our main objectives were the following 1) to assess the usefulness of biochemical parameters as predictors of an individual's physiological condition; 2) to determine if a relationship existed between serum biochemistry and serostatus such that rabbits in poorer condition are more likely to be seropositive for MV and RHDV; and 3) to establish baseline values for biochemical parameters of rabbits with different serostatus in wild rabbit populations.

2. Material and methods

2.1.1. Ethics statement

All animal experimentation was carried out in accordance with Spanish and European regulations (Law 32/2007, R.D. 1201/2005, and Council Directive 2010/63/EU, R.D. 53/2013, ECC/566/2015).

2.1.2. Study site

The study was conducted in Hornachuelos Natural Park (100–700 m a.s.l.), which is located in a mountainous area in the southwestern Iberian Peninsula (37°49′ N, 5°15′ W). The climate is Mediterranean, with hot, dry summers and wet, mild winters. Three enclosures were built and used as breeding zones for rabbits. The primary objective was to increase local rabbit abundance to boost the numbers of endangered predators. The three enclosures (E1:3.8 ha; E2: 4.1 ha; E3:2.9 ha) were surrounded by 2.5-m-high chain-link fence to prevent rabbit emigration and to exclude terrestrial predators (Rouco et al., 2008). Within the enclosures, 30 artificial warrens were constructed; they followed a regular distribution pattern. Water and food pellets were supplied *ad libitum*, and grasses were sown to increase the availability of fresh food.

2.1.3. Sampling

From autumn 2008 to spring 2010, we conducted seven live-trapping sessions in each enclosure. Rabbits were captured using cage traps placed in the proximity of each warren, as described by Bertó-Moran et al. (2013). This methodology resulted in the capture of about 50–60% of the rabbits occupying each warren on any given night (Rouco et al., 2011).

At the trap site, captured animals were marked with individually numbered ear tags and their sex and mass were recorded. Females and males weighing >750 g and 850 g, respectively, were considered to be adults (Villafuerte et al., 1994; Alves and Moreno, 1996).

To characterize biochemical parameters and antibody concentrations, blood samples (1–2.5 ml) were collected by venipuncture of the auricular marginal vein. The blood samples were kept at cold (4 °C approximately, without direct contact to ice), then transported to the field laboratory where they were immediately centrifuged in Eppendorf tubes. The serum obtained was stored at −80 °C until further analysis. (Evans, 2008; Maceda-Veiga et al., 2015)

2.1.4. Biochemical and immunological analyses

We processed the serum samples using a COBAS INTEGRA 400 plus analyzer (Productos Roche España, Madrid, Spain). We determined the concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BILI), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), urea (BUN), creatinine (CREA), albumin (ALB), and total proteins (TP). These biochemical parameters are indicators of organ function, protein metabolism, and nutritional status, which means they should be good proxies for physiological condition (Harder and Kirkpatrick, 1994; Stirrat, 2003). Since they were expressed in different units, they were transformed prior to analysis to enable comparisons.

Serum concentrations of anti-MV and anti-RHDV antibodies were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits; the diagnostic techniques recommended by the World Organization for Animal Health were used (OIE, 2012), and we strictly followed the manufacturer instructions. To measure anti-MV antibodies, sera were diluted 1:40, and a relative immunity index (RI) was obtained. It was defined as a coefficient between the optical density of controls (positive and negative) and that of sampled individuals. RI values ranged from 1 to 10. All rabbits with an RI > 2 were considered to be antibody positive (CIV TEST CUNI MIXOMATOSIS, HIPRA Laboratories, Girona, Spain). To measure anti-RHDV antibodies, the INGEZIM kit for rabbits (INGENASA Laboratories, Madrid, Spain) was used. Sera were screened using dilutions of 1:200, 1:400, 1:800, and 1:1600. Samples with optical densities >0.3 were considered to be antibody positive, since such antibody concentrations should be sufficient to confer protection against the disease (see Bertó-Moran et al., 2013). The test's specificity and sensitivity were 83.1% and 98.5%, respectively, and there was a 93% correspondence with the reference technique (OIE, 2012).

Biochemical and immunological analyses were performed by the Physiological Ecology Laboratory of the Doñana Biological Station -CSIC (Seville, Spain).

2.1.5. Data analysis

All statistical analyses were performed using R version 3.0.1 (R Core Team, 2013). Employing generalized linear mixed models (GLMM, glmer function, lme4 package) with a binomial distribution and a logit link function, we tested the relationships between the different biochemical parameters and serostatus for individuals in the three enclosures. To reduce heterogeneity, we limited our analyses to adults. Three sets of analyses were performed: 1) using rabbits seropositive for MV; 2) using rabbits seropositive for RHDV and 3) using rabbits

seropositive for both MV and RHDV. To avoid possible confounding effects, in all the analyses, we considered that individuals were seronegative only if they had neither anti-MV nor anti-RHDV antibodies. Correlations among biochemical parameters were tested, and ALT, GGT, BILI, CREA, BUN, and TP were retained as predictor variables in the subsequent analyses. Serostatus was the response variable. We also included sex and rabbit density as predictor variables in the models. Some individuals were sampled more than once by chance. To account for the increase in type I error (rejection of the null hypothesis when it is true) due to pseudoreplication (Hurlbert, 1984), we included the following random variable: capture session nested within individual identity nested within enclosure number.

Prior to running the analyses, all the numeric predictor variables were scaled (except for "sex") using the scale function so that their relative importance could be compared. We selected the best-fit models via backward stepwise selection (anova function with maximum likelihood, Crawley 2012; $p < 0.05$ as the threshold value). Each of the final models contained only the significant predictors.

3. Results

Through the course of the study, we got samples from 720 adult rabbits (274, 242, and 204 rabbits in E1, E2, and E3, respectively). A total of 346 samples were seropositive only for MV, 101 samples were seropositive only to RHDV, 200 samples were seropositive to both, MV and RHDV, and 245 samples were seronegative. Some individuals were sampled more than once and not always had the same antibody titre that is why the number of samples obtained does not match with the total number of individual animals handled.

None of the biochemical parameters analyzed were significantly associated with MV or RHDV serostatus. The only significant relationships we found were a positive association between rabbit density and MV seropositivity ($p < 0.001$; Table 1) and a negative association between GGT levels and seropositivity to both viruses ($p < 0.05$; Table 1).

Each enclosure displayed different seroprevalence patterns (Fig. 1). In E1, the percentage of rabbits seropositive for MV, RHDV, or both remained fairly constant over time. More specifically, MV seroprevalence was high for most of the trapping sessions. In contrast, RHDV seroprevalence was low; it peaked at 24.4% in session 3 (Fig. 1). In E2, the percentage of seronegative rabbits was generally higher than in E1 and E3, with values reaching a maximum of 63.8 and 69.4% in sessions 1 and 2. MV seroprevalence increased from session 3 to session 7, whereas the percentage of seronegative rabbits clearly declined (Fig. 1). Remarkably, no rabbits were seropositive for RHDV in session 7. In E3, there was a higher percentage of individuals that were seropositive for both viruses, as compared to E1 and E2. It was also the enclosure with the lowest percentage of seronegative rabbits; this value climbed as high as 55.6% in session 4 (Fig. 1). Notably, there were no seronegative rabbits in sessions 1 and 7. Rabbits seropositive for MV and for RHDV were observed in every session, but their percentages were rather low. RHDV seroprevalence peaked in all three enclosures in session 3 (Fig. 1).

In general, the ranges of values observed for the biochemical parameters remained fairly consistent, although some noticeable changes in certain parameters occurred during certain capture sessions (Fig. 2). In E1, most of the biochemical parameters had relatively constant values, but GGT and BILI fluctuated slightly. The pattern in E2 was more heterogeneous. Almost all the parameters varied somewhat, except for BUN, TP, ALB, and BILI. In the case of the transaminases—ALT, AST, and GGT—maximum values occurred in sessions 2, 5, and 7 (Fig. 2). CREA levels were fairly constant over time but hit a low in session 3, which coincided with the minimum values for the transaminases (Fig. 2). Blood biochemistry patterns were most distinct in E3. As in E2, BUN, TP, ALB, and BILI varied little while the transaminases and CREA fluctuated dramatically (Fig. 2). ALT and AST followed parallel patterns, both peaking in sessions 4, 6, and 7 and dropping to their minimum values in session 2 (Fig. 2). GGT presented an irregular pattern—levels were highest in session 4 and dipped down in sessions 3, 5, and 7. While CREA tended to remain constant, it dropped sharply after peaking in session 5 (Fig. 2).

Table 2 provides the means for the different biochemical parameters for the different enclosures and seropositivity classes; it also gives more detailed information related to the aforementioned patterns.

4. Discussion

To our knowledge, this is the first study conducted in the field to address the relationship between MV and RHDV seropositivity and the physiological status of wild European rabbits using large numbers of animals and in the context of a long-term monitoring program.

In light of the results, we found limited evidence for an association between blood biochemistry and serostatus in wild European rabbit populations. The only significant relationship we observed was that rabbits seropositive for both MV and RHDV had lower GGT concentrations (Table 1). However, the lack of significant findings might be due to spurious results generated by data heterogeneity and the presence of confounding variables.

One major methodological handicap is the scarcity of data on wild rabbit populations. Most studies dealing with myxomatosis and RHD have focused on disease pathology and epidemiology in domestic rabbits. Consequently, most of the information currently available has been obtained using rabbits reared under laboratory conditions (Calvete et al., 2002; Calvete et al., 2005; Cabezas et al., 2006; Kerr, 2012). However, physiological data for domestic rabbits is not directly comparable to that for wild rabbits since major differences exist in genetics, environmental contexts, breeding conditions, individual responsiveness, and even laboratory processes and techniques. In addition, laboratory rabbits usually develop physiological problems and specific pathologies as a result of living in captivity. These limitations aside, our results suggest that myxomatosis and RHD have declined in severity because they have become endemic in the Iberian Peninsula (Ross, 1986; Ross et al., 1989; Marchandeau and Boucraut-Baralon, 1999; Calvete et al., 2002; Marchandeau et al., 2014). Endemic diseases have strong initial effects and cause high mortality rates in afflicted populations. However, the individuals that survive experience constant

Table 1
Results for the generalized mixed models for each dataset (i.e., MV: rabbits seropositive for myxoma virus; RHDV: rabbits seropositive for rabbit hemorrhagic disease virus; MV & RHDV: rabbits seropositive for both viruses). Coefficient estimates (β), estimated standard errors (SE), and p-values (p) are listed.* indicates statistically significant value, P<0.005

	MV			RHDV			MV & RHDV		
	B	SE	p	β	SE	p	β	SE	p
ALT	0.2042	0.1241	0.09433	0.1940	0.1646	0.2486	0.2391	0.1362	0.08241
GGT	0.01504	0.11533	0.8961	−0.06207	0.17053	0.7131	−0.3031	0.1599	0.0491*
BILI	−0.07875	0.10509	0.453	−0.2079	0.1636	0.192	−0.1320	0.1427	0.3639
CREA	−0.04941	0.10756	0.6478	−0.03273	0.17178	0.8467	−0.1195	0.1651	0.4781
BUN	0.04409	0.10948	0.686	−0.01682	0.14124	0.9006	0.07518	0.11388	0.5182
ALB	−0.04380	0.11618	0.7065	0.02039	0.16918	0.8993	0.08296	0.14723	0.5847
density	0.3728	0.1112	0.000804*	−0.03413	0.17652	0.8461	0.01127	0.14411	0.9383
Sex	0.2173	0.2155	0.3112	0.10556	0.29962	0.7248	0.3539	0.2554	0.1703

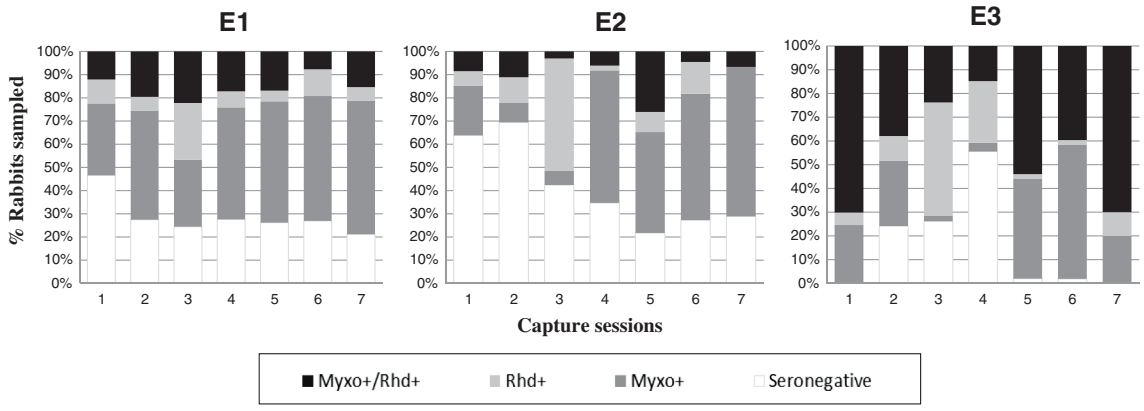

Fig. 1. Variation in MV and RHDV seroprevalence in rabbit populations (E1, E2, and E3) over the two-year study period.

reinfections over time, ultimately leading to high immunity levels within populations. As a result, individuals become partly protected and most show mild clinical symptoms throughout the year. The pathogen can then be said to be in permanent circulation and to have become enzootic (Calvete et al., 2002; Cooke, 2002; Fouchet et al., 2008). This state of affairs is consistent with our results (Fig. 1). Although the three enclosures exhibited some distinct differences, in general, there were always some individuals seropositive for MV, RHDV, or both throughout the study period. This finding suggests that the two viruses are now endemic in the study populations. It is also worth noting the fluctuating percentage of seronegative rabbits seen in E3: there were no seronegatives at the beginning or at the end of the study period. In E2, no RHDV-seropositive rabbits were found in the last capture session. Individuals with severe RHDV infections might have died, leaving no seropositives in the population; consequently, new outbreaks may result in high mortality rates. This pattern might be linked to the severity of RHD and its relatively more recent arrival, as compared to myxomatosis.

When we looked at the results for rabbit biochemistry and serostatus in tandem for the different enclosures, we observed that both were highly homogenous in E1. In E2, transaminases and CREA peaked in session 2, which was when the percentage of seronegative rabbits was the highest. The number of seronegative rabbits declined over subsequent sessions, while rabbits seropositive for MV, for RHDV, and for both became more abundant. One possible explanation is that E2 rabbits were exposed to the viruses around the time of session 2 (there were a number of outbreaks that season, as described in the literature [i.e., Calvete et al., 2002]), which is suggested by the session 2 peak in transaminases. In sessions 3 and 4, the number of seropositive rabbits increased and both the transaminases and CREA dropped to their minimum values.

This result lends support to the idea that rabbits that have been exposed to the viruses, and that consequently develop immunity, are likely to return to basal biochemical parameter values.

In E3, transaminases peaked in session 4, which is also when the number of seronegative rabbits was highest. In the subsequent capture sessions (sessions 5 and 6), the percentage of seronegative individuals declined sharply while the number of individuals seropositive for MV, RHDV, or both climbed. This pattern probably resulted from a high incidence of the diseases in session 4 and earlier. The population's exposure to the viruses can be seen in the increase in transaminases in session 4, which is when they reached maximum levels. In sessions 5, 6, and 7, after rabbits had become seropositive, the transaminases were close to their minimum levels, suggesting that immune (seropositive) rabbits tended to return to basal parameter levels.

As in E2, in E3 there were large numbers of seronegative rabbits in sessions 2 and 3, just before transaminases peaked in session 4, which likely signaled the beginning of an endemic disease cycle.

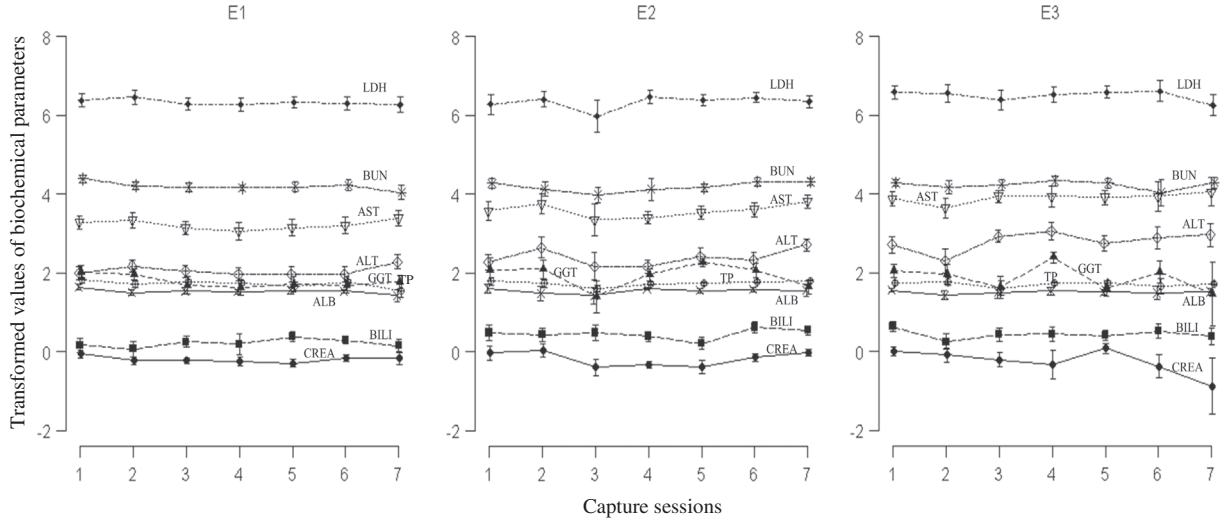

Fig. 2. Transformed values (mean ± SE) of biochemical parameters for each capture session in the three study enclosures (E1, E2, and E3). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin (BILI), lactate dehydrogenase (LDH), gamma glutamyltransferase (GGT), urea (BUN), creatinine (CREA), albumin (ALB), and total proteins (TP).

Table 2
Blood biochemistry of wild European rabbits in the three study enclosures (E1, E2 and E3); rabbits are grouped by serostatus (Seronegative, seropositive for myxoma virus [Myxo +], seropositive for rabbit hemorrhagic disease virus [Rhd +], and seropositive for both viruses [Myxo +/Rhd +]). Values correspond to the mean ± SE.

	E1				E2				E3			
Parameter (units)	Seronegative	Myxo +	Rhd +	Myxo +/Rhd +	Seronegative	Myxo +	Rhd +	Myxo +/Rhd +	Seronegative	Myxo +	Rhd +	Myxo +/Rhd +
ALT (U/L)	9 ± 0.7	8.5 ± 0.9	9.6 ± 0.5	10.1 ± 0.9	12.6 ± 0.8	14.7 ± 1.8	13.8 ± 1.0	15.1 ± 2.0	19.6 ± 2.1	20 ± 2.0	21 ± 1.4	20.3 ± 1.2
AST (U/L)	32.2 ± 3.2	31.4 ± 3.2	31.9 ± 1.9	38.1 ± 5.0	44.8 ± 3.0	46.4 ± 6.3	48.3 ± 4.1	39.8 ± 3.9	66 ± 9.1	56.8 ± 6.1	60.8 ± 4.0	65.3 ± 5.1
GGT (U/L)	6.9 ± 0.6	7 ± 0.7	7.4 ± 0.4	7.1 ± 0.6	9.2 ± 0.6	8 ± 1.1	8.4 ± 0.5	8 ± 0.8	9.2 ± 0.9	9.3 ± 0.9	10.3 ± 1.3	7.4 ± 0.4
BILI (μmol/L)	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	1.7 ± 0.2	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 0.1
CREA (mg/dl)	0.8 ± 0.04	0.8 ± 0.07	0.8 ± 0.02	0.8 ± 0.05	0.9 ± 0.04	0.8 ± 0.07	0.8 ± 0.05	0.9 ± 0.1	1 ± 0.2	0.9 ± 0.1	1 ± 0.1	0.9 ± 0.05
BUN (mg/dl)	73.7 ± 4.8	76.2 ± 7.9	74 ± 2.2	71.9 ± 3.4	70.4 ± 2.6	66 ± 4.9	77.7 ± 2.3	73.2 ± 5.7	76.5 ± 5.1	77.7 ± 3.3	72.4 ± 3.3	79.8 ± 3.4
LDH (U/L)	643.1 ± 43.5	647.7 ± 34	697.8 ± 44.0	697.4 ± 64.6	670.5 ± 37.9	582.9 ± 52.4	703 ± 36.9	740 ± 91.8	890 ± 98.0	743.2 ± 95.7	918.9 ± 73.2	862.4 ± 63.5
ALB (g/dl)	4.8 ± 0.1	5 ± 0.2	4.7 ± 0.1	4.7 ± 0.1	4.9 ± 0.1	4.5 ± 0.2	4.9 ± 0.1	5 ± 0.1	4.5 ± 0.2	4.7 ± 0.1	4.7 ± 0.1	4.6 ± 0.1
TP (g/dl)	5.7 ± 0.2	6.1 ± 0.4	5.7 ± 0.1	6 ± 0.4	5.8 ± 0.1	5.4 ± 0.2	5.8 ± 0.1	5.7 ± 0.3	5.4 ± 0.2	5.7 ± 0.1	5.8 ± 0.1	5.8 ± 0.1

Of the biochemical parameters studied, the transaminases (ALT, AST, and GGT) were clearly the most variable for all three enclosures. This pattern may reflect the impaired hepatic function seen in rabbits infected with MV and/or RHDV.

In addition to the shortcomings mentioned above, the lack of significant findings puts into question the utility of biochemical parameters in assessing the physiological condition of European rabbits. As is clear from the literature, serum biochemistry might be influenced by a variety of factors, including rabbit handling and sampling procedures, fieldwork conditions, and animal nutritional and health status at the time of sampling (Calvete et al., 2005; Cabezas et al., 2006). Furthermore, there is individual-level variation in immune and physiological responses as a result of trade-offs between environmental conditions and life-history traits (e.g., developmental, physiological, genetic, and immunological traits) (Ardia et al., 2011). Therefore, alternative indicators such as concentrations of specific immunoglobulins (e.g., IgM or IgG) or cellular oxidative stress markers could provide more complete and precise information.

As discussed above, confounding variables that were not accounted for in our analyses could be skewing our results. Such variables could include the following: (1) rabbit age; (2) outbreak timing; (3) the ELISA seropositivity thresholds; (4) the response speed of biochemical parameters; and (5) the lack of reference values for wild rabbits.

One major factor could be rabbit age. In this study, we estimated age based on mass. Although this approach can separate adult rabbits from non-adult rabbits, it cannot reveal a rabbit's precise age. Knowing a rabbit's age could be important because as rabbits get older, their probability of being infected by a wide variety of potentially serious pathogens like MV or RHDV increases, as do antibody levels (Marchandeau et al., 1995; Parkes et al., 2002; Parkes et al., 2008). Furthermore, a rabbit's innate responsiveness changes over its lifetime, which means that individuals of different ages will have different biochemical profiles and immunological experience.

Outbreak timing is also important but difficult to characterize. Myxomatosis and RHD outbreaks show some seasonal and geographic variation (Mutze et al., 2008; Mutze et al., 2010; Abrantes et al., 2012). More specifically, the occurrence of epizootics might vary across years and even among populations as a result of delayed breeding and variable climatic conditions, which can affect the abundance and activity of the pathogens' vectors. Determining the moment of infection is nearly impossible, so outbreak timing is only approximate.

As mentioned above, wild rabbits are naturally exposed to a wide variety of pathogens, whereas laboratory rabbits are artificially infected with a smaller selection of them. The ELISA techniques that we used to determine MV and RHDV seropositivity were developed using European rabbits kept under laboratory conditions. It may be that applying such seropositivity thresholds to wild rabbits could yield false positives and cross-reactions since laboratory rabbits are exposed to fewer pathogen species and thus have lower threshold antibody concentrations than wild rabbits (Kerr, 1997).

Finally, the response speed of biochemical parameters must be accounted for. Serum biochemistry changes are relatively transient, as demonstrated by several studies in which rabbits were artificially infected with pathogens (Ferreira et al., 2004). Rabbits show an initial physiological response to infection, but if they do not die, any changed biochemical parameters revert to their basal values. Nevertheless, such shifts are likely to go undetected in the wild.

In conclusion, it will be important to carry out further research that explores straightforward, reliable indices that can be used to assess the physiological condition of individuals in target wildlife populations. Selecting the right methods and biochemical parameters is essential if we wish to more rapidly detect and control diseases in wild species, which would help improve management and conservation programs.

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References

Abrantes, J., Van Der Loo, W., Le Pendu, J., Esteves, P.J., 2012. Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review. Vet. Res. 43, 12.
Alves, P., Moreno, S., 1996. Estudo da reprodução do coelho-bravo (*Oryctolagus cuniculus*) em Portugal. Rev. Florestal. 9, 149–166.
Ardia, D.R., Parmentier, H.K., Vogel, L.A., 2011. The role of constraints and limitation in driving individual variation in immune response. Funct. Ecol. 25, 61–73.
Beldomenico, P.M., Telfer, S., Gebert, S., Lukomski, L., Bennett, M., Begon, M., 2008. Poor condition and infection: a vicious circle in natural populations. Proc. R. Soc. Lond. B Biol. Sci. 275, 1753–1759.
Bertó-Moran, A., Pacios, I., Serrano, E., Moreno, S., Rouco, C., 2013. Coccidian and nematode infections influence prevalence of antibody to myxoma and rabbit hemorrhagic disease viruses in European rabbits. J. Wildl. Dis. 49, 10–17.
Cabezas, S., Calvete, C., Moreno, S., 2006. Vaccination success and body condition in the European wild rabbit: applications for conservation strategies. J. Wildl. Manag. 70, 1125–1131.
Calvete, C., Estrada, R., Villafuerte, R., Osácar, J.J., Lucientes, J., 2002. Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits. Vet. Rec. 150, 776–781.
Calvete, C., Angulo, E., Estrada, R., Moreno, S., Villafuerte, R., 2005. Quarantine length and survival of translocated European wild rabbits. J. Wildl. Manag. 69, 1063–1072.
Chandra, R.K., Newberne, P.M., 1977. Interactions of nutrition, infection, and immune response in animals. Nutrition, Immunity, and Infection. Springer, US, pp. 127–180.
Collinge, S.K., Ray, C., 2006. Disease Ecology: Community Ecology and Pathogen Dynamics. Oxford University Press.
Cooke, B.D., 2002. Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations. Rev. Sci. Tech. 21, 347–358.
Crawley, M.J., 2012. Analysis of Variance, in The R Book, second ed. John Wiley & Sons, Ltd, Chichester, UK http://dx.doi.org/10.1002/9781118448908.ch11.
Deem, S.L., Karesh, W.B., Weisman, W., 2001. Putting theory into practice: wildlife health in conservation. Conserv. Biol. 15, 1224–1233.
Dobson, A.P., Hudson, P.J., 1986. Parasites, disease and the structure of ecological communities. Trends Ecol. Evol. 1, 11–15.
Evans, G.O., 2008. Animal Hematotoxicology: A Practical Guide for Toxicologists and Biomedical Researchers. CRC Press.
Fenner, F., Woodroofe, G.M., 1953. The pathogenesis of infectious myxomatosis: the mechanism of infection and the immunological response in the European rabbit (*Oryctolagus cuniculus*). Br. J. Exp. Pathol. 34, 400.
Ferreira, P.G., Costa-e-Silva, A., Monteiro, E., Oliveira, M.J.R., Aguas, A.P., 2004. Transient decrease in blood heterophils and sustained liver damage caused by calicivirus infection of young rabbits that are naturally resistant to rabbit haemorrhagic disease. Rev. Vet. Sci. 76, 83–94.
Fouchet, D., Guitton, J.S., Marchandeau, S., Pontier, D., 2008. Impact of myxomatosis in relation to local persistence in wild rabbit populations: the role of waning immunity and the reproductive period. J. Theor. Biol. 250, 593–605.
Franzmann, A.W., Schwartz, C.C., 1988. Evaluating condition of Alaskan black bears with blood profiles. J. Wildl. Manag. 63–70.
Gershwin, M.E., Beach, R.S., Hurley, L.S., 1985. Nutrition and Immunity. Academic, Orlando, USA, FL.
Harder, J.D., Kirkpatrick, R.L., 1994. Physiological Methods in Wildlife Research. Research and Management Techniques for Wildlife and Habitats. 5th edition. The Wildlife Society, Bethesda, Maryland, USA, pp. 275–306.
Hellgren, E.C., Vaughan, M.R., Kirkpatrick, R.L., 1989. Seasonal patterns in physiology and nutrition of black bears in Great Dismal Swamp, Virginia-North Carolina. Can. J. Zool. 67, 1837–1850.
Hellgren, E.C., Rogers, L.L., Seal, U.S., 1993. Serum chemistry and hematology of black bears: physiological indices of habitat quality or seasonal patterns? J. Mammal. 74, 304–315.
Hudson, P.J., Dobson, A.P., Lafferty, K.D., 2006. Is a healthy ecosystem one that is rich in parasites? Trends Ecol. Evol. 21, 381–385.
Hurlbert, S.H., 1984. Pseudoreplication and the design of ecological field experiments. Ecol. Monogr. 54 (2), 187–211.
Kerr, P.J., 1997. An ELISA for epidemiological studies of myxomatosis: persistence of antibodies to myxoma virus in European rabbits (*Oryctolagus cuniculus*). Wildl. Res. 24, 53–65.
Kerr, P.J., 2012. Myxomatosis in Australia and Europe: a model for emerging infectious diseases. Antivir. Res. 93, 387–415.
Kerr, P.J., Donnelly, T.M., 2013. Viral infections of rabbits. Vet. Clin. North Am. Exot. Anim. Pract. 16, 437–468.
Liu, S.J., Xue, H.P., Pu, B.Q., Qian, N.H., 1984. A new viral disease in rabbits. Anim. Husb. Vet. Med. 16, 253–255.
Maceda-Veiga, A., Figuerola, J., Martínez-Silvestre, A., Viscor, G., Ferrari, N., Pacheco, M., 2015. Inside the Redbox: applications of haematology in wildlife monitoring and ecosystem health assessment. Sci. Total Environ. 514, 322–332.
Marchandeau, S., Boucraut-Baralon, C., 1999. Épidémiologie de la myxomatose et des caliciviroses apparentées à la VHD dans une population sauvage de lapins de garenne (*Oryctolagus cuniculus*). Gibier faune sauvage 16 (1), 65–80.
Marchandeau, S., Guenezan, M., Chantal, J., 1995. Utilisation de la croissance pondérale pour la détermination de l'âge de jeunes lapins de garenne (*Oryctolagus cuniculus*). Gibier faune sauvage 12 (4), 289–302.
Marchandeau, S., Pontier, D., Guitton, J.S., Letty, J., Fouchet, D., Aubineau, J., Bertagnoli, S., 2014. Early infections by myxoma virus of young rabbits (*Oryctolagus cuniculus*) protected by maternal antibodies activate their immune system and enhance herd immunity in wild populations. Vet. Res. 45, 1–9.
Milner, J.M., Stien, A., Irvine, R.J., Albon, S.D., Langvatn, R., Ropstad, E., 2003. Body condition in Svalbard reindeer and the use of blood parameters as indicators of condition and fitness. Can. J. Zool. 81, 1566–1578.
Møller, A.P., Christe, P., Erritzøe, J., Mavarez, J., 1998. Condition, disease and immune defence. Oikos 301–306.
Morner, T., Obendorf, D.L., Artois, M., Woodford, M.H., 2002. Surveillance and monitoring of wildlife diseases. Rev. Sci. Tech. 21 (1), 67–76.
Mutze, G., Bird, P., Cooke, B., Henzell, R., 2008. Geographic and seasonal variation in the impact of rabbit haemorrhagic disease on European rabbits, *Oryctolagus cuniculus*, and rabbit damage in Australia. Lagomorph Biology. Springer, Berlin Heidelberg, pp. 279–293.
Mutze, G., Kovaliski, J., Butler, K., Capucci, L., McPhee, S., 2010. The effect of rabbit population control programmes on the impact of rabbit haemorrhagic disease in south-eastern Australia. J. Appl. Ecol. 47, 1137–1146.
Nelson, R.J., Demas, G.E., 1996. Seasonal changes in immune function. Q. Rev. Biol. 511–548.
OIE (World Organisation for Animal Health), 2012. Technical Disease Cards. (Accessed September 2012) http://www.oie.int/en/ourscientific-expertise/specific-information-andrecommendations/technical-disease-cards/.
Parkes, J.P., Norbury, G.L., Heyward, R.P., Sullivan, G., 2002. Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997–2001. Wildl. Res. 29, 543–555.
Parkes, J.P., Glentworth, B., Sullivan, G., 2008. Changes in immunity to rabbit haemorrhagic disease virus, and in abundance and rates of increase of wild rabbits in Mackenzie Basin, New Zealand. Wildl. Res. 35, 775–779.
Pedersen, A.B., Fenton, A., 2007. Emphasizing the ecology in parasite community ecology. Trends Ecol. Evol. 22, 133–139.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ross, J., 1986. The influence of myxomatosis in regulating rabbit numbers. Population Dynamics and Epidemiology of Territorial Animals. 62, pp. 433–441.
Ross, J., Tittensor, A.M., Fox, A., Sanders, M.F., 1989. Myxomatosis in farmland rabbit populations in England and Wales. Epidemiol. Infect. 103, 333–357.
Rouco, C., Ferreras, P., Castro, F., Villafuerte, R., 2008. The effect of exclusion of terrestrial predators on short-term survival of translocated European wild rabbits. Wildl. Res. 35, 625–632.
Rouco, C., Villafuerte, R., Castro, F., Ferreras, P., 2011. Effect of artificial warren size on a restocked European wild rabbit population. Anim. Conserv. 14, 117–123.
Santoro, S., Pacios, I., Moreno, S., Bertó-Morán, A., Rouco, C., 2014. Multi-event capture–recapture modeling of host–pathogen dynamics among European rabbit populations exposed to myxoma and Rabbit Hemorrhagic Disease Viruses: common and heterogeneous patterns. Vet. Res. 45, 39.
Schroeder, M.T., 1987. Blood chemistry, hematology, and condition evaluation of black bears in northcoastal California. Bears: Their Biology and Management, pp. 333–349.
Stanford, M.M., Werden, S.J., McFadden, G., 2007. Myxoma virus in the European rabbit: interactions between the virus and its susceptible host. Vet. Res. 38, 299–318.
Stirrat, S.C., 2003. Body condition and blood chemistry of agile wallabies (*Macropus agilis*) in the wet–dry tropics. Wildl. Res. 30, 59–67.
Thomas, F., et al., 2005. Parasites and Ecosystems. Oxford University Press.
Tompkins, D.M., Begon, M., 1999. Parasites can regulate wildlife populations. Parasitol. Today 15, 311–313.
Viggers, K.L., Lindenmayer, D.B., Spratt, D.M., 1993. The importance of disease in reintroduction programmes. Wildl. Res. 20, 687–698.
Villafuerte, R., Calvete, C., Gortázar, C., Moreno, S., 1994. First epizootic of rabbit hemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana National Park, Spain. J. Wildl. Dis. 30, 176–179.
Woodroffe, R., 1999. Managing disease threats to wild mammals. Anim. Conserv. 2, 185–193.
Xu, W.Y., 1991. Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation. Rev. Sci. Tech. 10, 393–408.

REFERENCES

Abrantes, J., Van Der Loo, W., Le Pendu, J., Esteves, P. J., 2012. **Rabbit haemorrhagic disease (RHD) and rabbit haemorrhagic disease virus (RHDV): a review**. Veterinary research, 43(1), 12.

Abrantes, J., Lopes, A. M., Dalton, K. P., Melo, P., Correia, J. J., Ramada, M., Alves, P. C., Parra, F., Esteves, P. J., 2013. **New variant of rabbit hemorrhagic disease virus, Portugal, 2012–2013**. Emerging infectious diseases, 19(11), 1900.

Alves, P., Moreno, S., 1996. **Estudo da reprodução do coelho-bravo (*Oryctolagus cuniculus*) em Portugal**. Revista Florestal, 9, 149-166.

Alves, P. C., Hackländer, K., 2008. **Lagomorph species: geographical distribution and conservation status. Lagomorph biology: evolution, ecology, and conservation** . Eds: Alves, P.C., Ferrand, N. and Hackländer, K., 395-405.

Anderson, D. R., Burham, K. P., White, G. A., 2001. **Kullback- Leibler information in resolving natural resource conflicts when definitive data exist**. Wildlife Society Bulletin, 1260–1270.

Apel, K., Hirt, H., 2004. **Reactive oxygen species: metabolism, oxidative stress, and signal transduction**. Annual Review of Plant Biology, 55, 373-399.

Aragão, H., 1927. **Sur un flogelle du latex de Manihot palmata, *Phytomonas francai* n. sp.** Compte Rendu de la Societe de Biologie., 1077, 1080.

Ardia, D. R., Parmentier, H. K., Vogel, L. A., 2011. **The role of constraints and limitation in driving individual variation in immune response**. Functional Ecology, 25(1), 61-73.

Argüello Villares, J. L., Llanos Pellitero, A., Pérez-Ordoyo García, L. I., 1989. **Enfermedad vírica hemorrágica del conejo en España**. Cunicultura, 14(77), 0017-22.

Armour, C. J., Thompson, H. V., 1955. Spread of myxomatosis in the first outbreak in Great Britain. Annals of applied Biology, 43(3), 511-518.

Arnason, A. N., 1973. **The estimation of population size, migration rates and survival in a stratified population**. Researches on population ecology, 15(2), 1-8.

Artois, M., Bengis, R., Delahay, R. J., Duchêne, M. J., Duff, J. P., Ferroglio, E., Gortazar, C., Hutchings, M. R., Kock, R. A., Leighton, F. A., Mörner, T., Smith, G. C., 2009. **Wildlife disease surveillance and monitoring**. In **Management of disease in wild mammals**, 187-213. Springer Japan.

Asgari, S., Hardy, J. R., Sinclair, R. G., Cooke, B. D., 1998. **Field evidence for mechanical transmission of rabbit haemorrhagic disease virus (RHDV) by flies (Diptera: Calliphoridae) among wild rabbits in Australia**. Virus research, 54(2), 123-132.

Atkinson, R. J., Rokas, A., Stone, G. N., 2007. **Longitudinal patterns in species richness and genetic diversity in European oaks and oak gallwasps**. Phylogeography of southern European refugia, 127-151.

Baily, J. L., Dagleish, M. P., Graham, M., Maley, M., Rocchi, M. S., 2014. **RHDV variant 2 presence detected in Scotland**. Veterinary Record, 174(16), 411-411.

Barrio, I. C., 2010. **The rabbit as an agricultural pest in its native distribution range**. PhD, Universidad de Castilla-La Mancha.

Barrio, I. C., Bueno, C. G., Tortosa, F. S., 2010. **Alternative food and rabbit damage in vineyards of southern Spain**. Agriculture, ecosystems and environment, 138(1), 51-54.

Beckman, K. B., Ames, B. N., 1998. **The free radical theory of aging matures**. Physiological Reviews, 78(2), 547-581.

Beldomenico, P. M., Telfer, S., Gebert, S., Lukomski, L., Bennett, M., Begon, M., 2008. **Poor condition and infection: a vicious circle in natural populations**. Proceedings of the Royal Society. Series B, Biological Sciences, 275(1644), 1753-1759.

Benskin, C. M., Wilson, K., Jones, K., Hartley, I. R., 2009. **Bacterial pathogens in wild birds: a review of the frequency and effects of infection**. Biological Reviews, 84(3), 349-373.

Bertó-Moran, A., Pacios, I., Serrano, E., Moreno, S., Rouco, C., 2013**. Coccidian and nematode infections influence prevalence of antibody to myxoma and rabbit hemorrhagic disease viruses in European rabbits**. Journal of wildlife diseases, 49(1), 10-17.

Best, S. M., Kerr, P. J., 2000. **Coevolution of host and virus: the pathogenesis of virulent and attenuated strains of myxoma virus in resistant and susceptible European rabbits**. Virology, 267(1), 36-48.

Biadi, F., Le Gall, A., 1993. **Le lapin de garenne: Vie, gestion et chasse d'un gibier authentique**. Paris: Hatier.

Biju-Duval, C., Ennafaa, H., Dennebouy, N., Monnerot, M., Mignotte, F., Soriguer, R. C., El Gaaïed, A., El Hili, A., Mounolou, J. C., 1991. **Mitochondrial DNA evolution in lagomorphs: origin of systematic heteroplasmy and organization of diversity in European rabbits**. Journal of Molecular Evolution, 33(1), 92-102.

Blanco, J. C., Villafuerte, R., 1993. **Factores ecológicos que influyen sobre las poblaciones de conejo. Efectos de la enfermedad hemorrágica vírica**. Unpublished report. TRAGSA, Madrid, Spain.

Blas-Aritio, L., 1970. **Vida y costumbre de los mustélidos Españoles**. Servicio de Pesca Continental, Caza y Parques Nacionales-Ministerio de Agricultura, Madrid.

Blasco, S., Torres, J., Feliu, C., Casanova, J. C., Miquel, J., Moreno, S., 1996. **The helminthfauna of *Oryctolagus cuniculus* (Linnaeus, 1758) in the Iberian Peninsula**. Faunistic and ecological considerations. Parasite, 3(4), 327-333.

Blázquez, M. C., Villafuerte, R., 1990. **Nesting of the Montpellier snake (*Malpolon monspessulanus*) inside rabbit warrens at Doñana National Park (SW Spain): phenology and a probable case of communal nesting**. Journal of Zoology 222(4), 692–693.

Boag, B., 1985. **The localised spread of virus-vector nematodes adhering to farm machinery**. Nematologica, 31(2), 234-235.

Boag, B., 1988. **Observations on the seasonal incidence of myxomatosis and its interactions with helminth parasites in the European rabbit (*Oryctolagus cuniculus*)**. Journal of Wildlife Diseases, 24(3), 450-455.

Boag, B., Lello, J., Fenton, A., Tompkins, D. M., Hudson, P. J., 2001. **Patterns of parasite aggregation in the wild European rabbit (*Oryctolagus cuniculus*)**. International Journal for Parasitology, 31(13), 1421-1428.

Boag, B., Hernandez, A. D., Cattadori, I. M., 2013. **Observations on the epidemiology and interactions between myxomatosis, coccidiosis and helminth parasites in a wild rabbit population in Scotland**. European journal of wildlife research, 59(4), 557-562.

Branco, M. S., 1995. **Contribuição para o estudo da genética bioquímica e populacional do coelho, *Oryctolagus cuniculus***. PhD. Universidade do Pôrto.

Branco, M., Ferrand, N., Monnerot, M., 2000. **Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome b gene**. Heredity, 85(4), 307-317.

Branco, M., Monnerot, M., Ferrand, N., Templeton, A. R., 2002. **Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested clade and mismatch analyses of mitochondrial DNA genetic variation.** Evolution, 56(4), 792-803.

Bruce, J. S., Twigg, L. E., Gray, G. S., 2004. **The epidemiology of rabbit haemorrhagic disease, and its impact on rabbit populations, in south-western Australia**. Wildlife Research, 31(1), 31-49.

Buege, J. A., Aust, S. D., 1978. **Microsomal lipid peroxidation**. Methods in Enzymology, 52, 302-10.

Bull, P. C., 1958. **Incidence of coccidia (Sporozoa) in wild rabbits, *Oryctolagus cuniculus* (L.) in Hawke's Bay, New Zealand**. New Zealand Journal of Science, 1, 289-328.

Bull, P. C., 1959. **A seasonal sex difference in the infestation of rabbits with the nematode *Trichostrongylus retortaeformis* (Zeder, 1800)**. Nature, 184 (4682), 281-282.

Bull, P. C., 1964. **Ecology of helminth parasites of the wild rabbit *Oryctolagus cuniculus* (L.) in New Zealand**., 158.

Burnham, K. P., Anderson, D. R., 2003. **Model selection and multimodel inference: a practical information-theoretic approach**. Springer Science and Business Media.

Butler, F. T., 1994. **Arthropod and helminth parasites from rabbits *Oryctolagus cuniculus* in south-west Ireland**. The Irish Naturalists' Journal, 24(10), 392-395.

Cabezas, S., Calvete, C., Moreno, S., 2006. **Vaccination success and body condition in the European wild rabbit: applications for conservation strategies**. Journal of Wildlife Management, 70(4), 1125-1131.

Cabezas, S., Moreno, S., 2007. **An experimental study of translocation success and habitat improvement in wild rabbits**. Animal Conservation, 10(3), 340-348.

Cabral, M. J., Almeida, J., Almeida, P. R., Dellinger, T., Ferrand de Almeida, N., Oliveira, M. E., Palmeirim, J. M., Queirós, A. I., Rogado, L., Santos-Reis, M., 2005. '**Livro Vermelho dos Vertebrados de Portugal**.' Instituto da Conservação da Natureza: Lisboa, Portugal.

Calvete, C., 2006a. **The use of immunization programs in wild populations: Modelling effectiveness of vaccination campaigns against rabbit hemorrhagic disease**. Biological Conservation, 130(2), 290-300.

Calvete, C., 2006b. **Modeling the effect of population dynamics on the impact of rabbit hemorrhagic disease**. Conservation Biology, 20(4), 1232-1241.

Calvete, C., Estrada, R., Villafuerte, R., Osácar, J. J., Lucientes, J., 2002. **Epidemiology of viral haemorrhagic disease and myxomatosis in a free-living population of wild rabbits**. Veterinary Record, 150(25), 776-781.

Calvete, C., Estrada, R., Angulo, E., Cabezas-Ruiz, S., 2004a. **Habitat factors related to wild rabbit conservation in an agricultural landscape**. Landscape Ecology, 19(5), 531-542.

Calvete, C., Estrada, R., Lucientes, J., Osacar, J. J., Villafuerte, R., 2004b. **Effects of vaccination against viral haemorrhagic disease and myxomatosis on long-term mortality rates of European wild rabbits**. Veterinary Record, 155, 388-391.

Calvete, C., Estrada, R., Osacar, J. J., Lucientes, J., Villafuerte, R., 2004c. **Short-term negative effects of vaccination campaigns against myxomatosis and viral hemorrhagic disease (VHD) on the survival of European wild rabbits**. Journal of Wildlife Management, 68(1), 198-205.

Calvete, C., Angulo, E., Estrada, R., Moreno, S., Villafuerte, R., 2005. **Quarantine length and survival of translocated European wild rabbits**. The Journal of Wildlife Management, 69(3), 1063-1072.

Calvete, C., Pelayo, E., Sampietro, J., 2006. **Habitat factors related to wild rabbit population trends after the initial impact of rabbit haemorrhagic disease**. Wildlife Research, 33(6), 467-474.

Capucci, L., Fusi, P., Lavazza, A., Pacciarini, M. L., Rossi, C., 1996. **Detection and preliminary characterization of a new rabbit calicivirus related to rabbit hemorrhagic disease virus but nonpathogenic**. Journal of Virology, 70(12), 8614-8623.

Carmagnol, F., Sinet, P. M., Jerome, H., 1983. **Selenium-dependent and non-selenium-dependent glutathione peroxidases in human tissue extracts**. Biochimica et Biophysica Acta, 759(1-2), 49-57.

Carneiro, M., Ferrand, N., Nachman, M. W., 2009. **Recombination and speciation: loci near centromeres are more differentiated than loci near telomeres between subspecies of the European rabbit (*Oryctolagus cuniculus*)**. Genetics, 181(2), 593-606.

Carvalho, J. C., Gomes, P., 2004. **Influence of herbaceous cover, shelter and land cover structure on wild rabbit abundance in NW Portugal**. Acta Theriologica, 49(1), 63-74.

Castillo-Rodríguez, C., Wittwer-Menge, F., Cerón, J. J., 2011. **Oxidative Stress** In **Veterinary Medicine**. Veterinary Medicine International, 2011, 1-1.

Cattadori, I. M., Albert, R., Boag, B., 2007. **Variation in host susceptibility and infectiousness generated by co-infection: the myxoma–*Trichostrongylus retortaeformis* case in wild rabbits**. Journal of the Royal Society Interface, 4(16), 831-840.

Cattadori, I. M., Boag, B., Hudson, P. J., 2008. **Parasite co-infection and interaction as drivers of host heterogeneity**. International journal for parasitology, 38(3), 371-380.

Cerván Carmona, C. M., Pardo Navarro, F., 1997. **Dispersión de semillas de retama (*Retama spaherocarpa*, L. Boiss) por el conejo (*Oryctolagus cuniculus*) en el centro de España**. Doñana Acta Vertebrata, 24(1-2), 143-154.

Chambert, T., Staszewski, V., Lobato, E., Choquet, R., Carrie, C., McCoy, K. D., Tveraa, T., Boulinier, T., 2012. **Exposure of black-legged kittiwakes to Lyme disease spirochetes: dynamics of the immune status of adult hosts and effects on their survival**. Journal of Animal Ecology, 81(5):986–995.

Chandra, R. K., Newberne, P. M., 1977. **Interactions of Nutrition, Infection, and Immune Response in Animals**. In: Nutrition, Immunity, and Infection, Springer US, 127-180.

Chapuis, J. L., 1990. **Comparation of the diets of two sympatric lagomorphs, *Lepus europaeus* (Pallas) and *Oryctolagus cuniculus* (L.) in an agroecosystem of the Ile-de-France.** Zeitschrift fur Säugertierkunde, 55, 176-185.

Chasey, D., 1994. **Possible origin of rabbit haemorrhagic disease in the United Kingdom**. The Veterinary Record, 135(21), 496–499.

Choquet, R., Reboulet, A. M., Lebreton, J. D., Gimenez, O., Pradel, R., 2005. **U-CARE 2.2 user's manual**. CEFE, Montpellier, France, 53.

Choquet, R., Lebreton, J. D., Gimenez, O., Reboulet, A. M., Pradel, R., 2009. **U-CARE: Utilities for performing goodness of fit tests and manipulating Capture–Recapture data**. Ecography, 32(6), 1071-1074.

Choquet, R., Nogue, E., 2011. **E-SURGE 1.8 User's Manual** (English Edition). CEFE, Montpellier, France.

Christensen, L. L., Selman, C., Blount, J. D., Pilkington, J. G., Watt, K. A., Pemberton, J. M., Reid, J. M., Nussey, D. H., 2015. **Plasma markers of oxidative stress are uncorrelated in a wild mammal**. Ecology and Evolution, 5(21), 5096-5108.

Chylinski, C., Boag, B., Stear, M. J., Cattadori, I. M., 2009**. Effects of host characteristics and parasite intensity on growth and fecundity of Trichostrongylus retortaeformis infections in rabbits**. Parasitology, 136(1), 117-123.

Clarkson, P. M., Thompson, H. S., 2000. **Antioxidants: what role do they play in physical activity and health?** The American Journal of Clinical Nutrition, 72, 637s-646s.

Cohen, G., Somerson, N. L., 1969. **Glucose-dependent secretion and destruction of hydrogen peroxide by *Mycoplasma pneumoniae***. Journal of Bacteriology, 98(2), 547-551.

Cohen, A. A., McGraw, K. J., 2009. **No simple measures for antioxidant status in birds: complexity in inter-and intraspecific correlations among circulating antioxidant types**. Functional Ecology, 23(2), 310-320.

Collinge, S. K., Ray, C., 2006. **Disease Ecology: Community Ecology and Pathogen** Dynamics, Oxford University Press.

Conn, P. B., Cooch, E. G., 2009. **Multistate capture–recapture analysis under imperfect state observation: an application to disease models**. Journal of Applied Ecology, 46(2), 486-492.

Cooch, E. G., Conn, P. B., Ellner, S. P., Dobson, A. P., Pollock, K. H., 2012. **Disease dynamics in wild populations: modeling and estimation: a review**. Journal of Ornithology, 152(2), 485-509.

Cooke, B. D., 2002. **Rabbit haemorrhagic disease: field epidemiology and the management of wild rabbit populations**. Revue Scientifique et Technique-Office International des Epizooties, 21(1), 347-358.

Cooke, B. D., Fenner, F., 2002. **Rabbit haemorrhagic disease and the biological control of wild rabbits, Oryctolagus cuniculus, in Australia and New Zealand**. Wildlife Research, 29(6), 689-706.

Corbet, G. B., 1994. **Taxonomy and origins**. In **The European rabbit. The history and Biology of a successful colonizer.** Eds: Tompson, H.V., King, C.M. Oxford: Oxford Science Publications, 1-6.

Costantini, D., 2008. **Oxidative stress in ecology and evolution: lessons from avian studies**. Ecology Letters, 11(11), 1238-1251.

Costantini, D., 2014. **Oxidative stress and hormesis in evolutionary ecology and physiology. A marriage between mechanistic and evolutionary approaches**. Berlin and Heidelberg. Springer, 1-38.

Costantini, D., Møller, A. P., 2009. **Does immune response cause oxidative stress in birds? A meta-analysis.** Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 153(3), 339-344.

Costantini, D., Verhulst, S., 2009. **Does high antioxidant capacity indicate low oxidative stress?** Functional Ecology, 23(3), 506-509.

Cosyns, E., Delporte, A., Lens, L., Hoffmann, M., 2005. **Germination success of temperate grassland species after passage through ungulate and rabbit guts**. Journal of Ecology, 93(2), 353-361.

Cotilla, I., Delibes-Mateos, M., Ramírez, E., Castro, F., Cooke, B. D., Villafuerte, R., 2010. **Establishing a serological surveillance protocol for rabbit hemorrhagic disease by combining mathematical models and field data: implication for rabbit conservation**. European journal of wildlife research, 56(5), 725-733.

Coudert, P., Licois, D., Drouet-viard, F., Provôt, F., 2000. **Coccidiosis.** In **Enfermedades del conejo**. Tomo II. Mundi-Prensa, Madrid, Spain, 219–234.

Cowan, D. P., 1985. **Coccidiosis in rabbits**. Population dynamics and epidemiology of territorial animals, 10.

Cowan, D. P., Garson, P. J., 1985. **Variations in the social structure of rabbit populations: causes and demographic consequences**. In **Behavioural Ecology: Ecological consequences of adaptive behaviour.** Eds: Sibly, R.M., and Smith, R.H. Blackwell Scientific Publications, Oxford, 537-555.

Cox, F. E. G., 2001**. Concomitant infections, parasites and immune responses**. Parasitology, 122(S1), S23-S38.

Crawley, M. J., 1990. **Rabbit grazing, plant competition and seedling recruitment in acid grassland**. Journal of Applied Ecology, 27(3), 803-820.

Crawley, M. J., Weiner, J., 1991. **Plant size variation and vertebrate herbivory: winter wheat grazed by rabbits**. Journal of Applied Ecology, 28(1), 154-172.

Cribb, A. E., Leeder, J. S., Spielberg, S. P., 1989. **Use of a microplate reader in an assay of glutathione reductase using 5, 50-dithiobis (2-nitrobenzoic acid).** Analytical Biochemistry: Methods in the Biological Sciences, 183(1), 195-196.

Dalton, K. P., Nicieza, I., Balseiro, A., Muguerza, M. A., Rosell, J. M., Casais, R., Álvarez, A. L., Parra, F., 2012. **Variant rabbit hemorrhagic disease virus in young rabbits, Spain**. Emerging infectious diseases, 18(12), 2009.

Daszak, P., Cunningham, A. A., Hyatt, A. D., 2000. **Emerging infectious diseases of wildlife - threats to biodiversity and human health**. Science, 287(5452), 443.

Deem, S. L., Karesh, W. B., Weisman, W., 2001. **Putting theory into practice: wildlife health in conservation**. Conservation Biology, 15(5), 1224-1233.

Delibes, M., Hiraldo, F., 1981. **The rabbit as prey in the Iberian Mediterranean ecosystem**. In **Proceedings of the World Lagomorph Conference**, August 1979. Eds: Myers, K. and MacInnes, C.D. Guelph University Press, Guelph, Ontario, 614-622.

Delibes-Mateos, M., 2006. **Relaciones entre los cambios poblacionales de conejo, la gestion cinégetica, el habitat y los depredadores. Implicaciones para la conservación** . PhD, Universidad de Castilla-La Mancha.

Delibes-Mateos, M., Redpath, S. M., Angulo, E., Ferreras, P., Villafuerte, R., 2007. **Rabbits as a keystone species in southern Europe**. Biological Conservation, 137(1), 149-156.

Delibes-Mateos, M., Delibes, M., Ferreras, P., Villafuerte, R., 2008. **Key role of European rabbits in the conservation of the Western Mediterranean basin hotspot**. Conservation Biology, 22(5), 1106-1117.

Delibes-Mateos, M., Rouco, C., Villafuerte, R., 2009a. **Can adult and juvenile European rabbits be differentiated by their pellet sizes?**. Acta oecologica, 35(2), 250-252.

Delibes-Mateos, M., Ferreras, P., Villafuerte, R., 2009b. **European rabbit population trends and associated factors: a review of the situation in the Iberian Peninsula**. Mammal Review, 39(2), 124-140.

Delibes-Mateos, M., Farfán, M. A., Olivero, J., Vargas, J. M., 2010. **Land-use changes as a critical factor for long-term wild rabbit conservation in the Iberian Peninsula**. Environmental Conservation, 37(2), 169-176.

Delibes-Mateos, M., Smith, A. T., Slobodchikoff, C. N., Swenson, J. E., 2011. **The paradox of keystone species persecuted as pests: a call for the conservation of abundant small mammals in their native range**. Biological Conservation, 144(5), 1335-1346.

Delibes-Mateos, M., Giergiczny, M., Caro, J., Viñuela, J., Riera, P., Arroyo, B., 2014. **Does hunters' willingness to pay match the best hunting options for biodiversity conservation? A choice experiment application for small-game hunting in Spain**. Biological Conservation, 177, 36-42.

Delibes-Mateos, M., Farfán, M. A., Rouco, C., Olivero, J., Márquez, A. L., Fa, J., Vargas, J. M., Villafuerte, R., 2017. **Agricultural damage by the European rabbit in Spain is promoted by transport infrastructure, not by natural vegetation**. Pest Management Science. doi:10.1002/ps.4658.

Dellafiore, C. M., Muñoz Vallés, S., Gallego Fernández, J. B., 2006. **Rabbits (*Oryctolagus cuniculus*) as dispersers of *Retama monosperma* seeds in a coastal dune system**. EcoScience, 13(1), 5–10.

Dendy, J., Mckillop, G., Fox, S., Western, G., Langton, S., 2004. **A field trial to assess the effects of rabbit grazing on spring barley**. Annals of applied biology, 145(1), 77-80.

Dobson, A. P., Hudson, P. J., 1986. **Parasites, disease and the structure of ecological communities**. Trends in Ecology and Evolution, 1(1), 11-15.

Dobson, A., Foufopoulos, J., 2001. **Emerging infectious pathogens of wildlife**. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 356(1411), 1001-1012.

Dowling, D. K., Simmons, L. W., 2009**. Reactive oxygen species as universal constraints in life-history evolution**. Proceedings of the Royal Society. Series B, Biological Sciences, 276, 1737-1745.

Dröge, W., 2002. **Free radicals in the physiological control of cell function**. Physiological Reviews, 82(1), 47-95.

Dunsmore, J. D., 1966a. **Nematode parasites of free-living rabbits, *Oryctolagus cuniculus* (L.), in eastern Australia. I. Variations in the numbers of *Trichostrongylus retortaeformis* (Zeder)**. Australian Journal of Zoology, 14 (4), 185-199.

Dunsmore, J. D., 1966b. **Nematode parasites of free-living rabbits, *Oryctolagus cuniculus* (L.), in eastern Australia. II. Variations in the numbers of *Graphidium strigosum* (Dujardin) Railliet and Henry**. Australian Journal of Zoology, 14 (4), 625-634.

Dunsmore, J. D., 1966c. **Nematode parasites of free-living rabbits, *Oryctolagus cuniculus* (L.), in eastern Australia. III. Variations in the numbers of *Passalurus ambiguas* (Rudolphi)**. Australian Journal of Zoology, 14 (4), 635-645.

Dunsmore, J. D., Dudzinski, M. L., 1968. **Relationship of numbers of nematode parasites in wild rabbits, *Oryctolagus cuniculus* (L.), to host sex, age, and season**. The Journal of Parasitology, 462-474.

Dunsmore, J. D., 1980. **The role of parasites in population regulation of the European rabbit (*Oryctolagus cuniculus*) in Australia**. In Proceedings of the Worldwide Furbearing Conference . Ed. Chapman, J.A. and, Pursley, D., 2, 654-669.

Eldridge, D. J., Simpson, R., 2002. **Rabbit (*Oryctolagus cuniculus* L.) impacts on vegetation and soils, and implications for management of wooded rangelands**. Basic and Applied Ecology, 3(1), 19-29.

Esteves, P. J., Alves, P. C., Ferrand, N., 2006. **O uso de marcadores genéticos na gestaõ e conservaçaõ de populações de coelho-bravo (*Oryctolagus cuniculus*)**. En: **Gestaõ e conservaçaõ de populações de coelho-bravo**. 11-30. Federaçao Alentejana de Caçadores (Eds.), 188.

Evans, G. O., 2008. **Animal hematotoxicology: a practical guide for toxicologists and biomedical researchers**. CRC Press.

Farrow, E. P., 1917. **On the ecology of the vegetation of Breckland: III. General effects of rabbits on the vegetation**. Journal of Ecology, 5(1), 1-18.

Faustino, C. R., Jennelle, C. S., Connolly, V., Davis, A. K., Swarthout, E. C., Dhondt, A. A., Cooch, E. G., 2004. ***Mycoplasma gallisepticum*** **infection dynamics in a house finch population: seasonal variation in survival, encounter and transmission rate**. Journal of Animal Ecology, 73(4), 651-669.

Fenner, F., Woodroofe, G. M., 1953. **The pathogenesis of infectious myxomatosis: the mechanism of infection and the immunological response in the European rabbit (*Oryctolagus cuniculus*)**. British Journal of Experimental Pathology, 34(4), 400.

Fenner, F., Ratcliffe, F. N., 1965. **Myxomatosis**. Cambridge University Press, Cambridge, England.

Fenner, F. J., White, D. O., 1976. **Medical virology** (No. 2nd edition). Academic Press Inc.(London) Ltd, 24/28 Oval Road, London NW1.

Fenner, F., Ross, J., 1994. Myxomatosis. In: Thompson, H.V., King, C.M. (Eds.), **The European Rabbit. The History and Biology of a Successful Colonizer**. Oxford University Press, Oxford, 205–240.

Fenton, E. W., 1940. **The influence of rabbits on the vegetation of certain hill-grazing districts of Scotland**. The Journal of Ecology, 28(2), 438-449.

Fernandez-de-Simon, J., Díaz-Ruiz, F., Cirilli, F., Tortosa, F. S., Villafuerte, R., Delibes-Mateos, M., Ferreras, P., 2011. **Towards a standardized index of European rabbit abundance in Iberian Mediterranean habitats**. European Journal of Wildlife Research, 57(5), 1091-1100.

Ferreira, C. C., Castro, F., Piorno, V., Barrio, I. C., Delibes-Mateos, M., Rouco, C., Mínguez, L. E., Aparicio, F., Blanco-Aguiar, J. A., Ramírez, E., Iriarte, C., Ríos-Saldaña, C. A., Cañadilla, J., Arias de Reyna, L., Ferreras, P., Alves, P. C., Villafuerte, R., 2015. **Biometrical analysis reveals major differences between the two subspecies of the European rabbit**. Biological Journal of the Linnean Society, 116(1), 106-116.

Ferreira, P. G., Costa-e-Silva, A., Monteiro, E., Oliveira, M. J. R., Aguas, A. P., 2004. **Transient decrease in blood heterophils and sustained liver damage caused by calicivirus infection of young rabbits that are naturally resistant to rabbit haemorrhagic disease**. Research in veterinary science, 76(1), 83-94.

Ferreira, P. G., Costa-e-Silva, A., Oliveira, M. J. R., Monteiro, E., Aguas, A. P., 2005. **Leukocyte–hepatocyte interaction in calicivirus infection: differences between rabbits that are resistant or susceptible to rabbit haemorrhagic disease (RHD)**.Veterinary immunology and immunopathology, 103(3), 217-221.

Ferreira, P. G., Costa-e-Silva, A., Monteiro, E., Oliveira, M. J. R., Águas, A. P., 2006. **Liver enzymes and ultrastructure in rabbit haemorrhagic disease (RHD)**. Veterinary research communications, 30(4), 393-401.

Ferreira, P. G., Dinis, M., Costa-e-Silva, A., Aguas, A. P., 2008. **Adult rabbits acquire resistance to lethal calicivirus infection by adoptive transfer of sera from infected young rabbits**. Veterinary immunology and immunopathology, 121(3), 364-369.

Ferrer, M., Negro, J. J., 2004. **The near extinction of two large European predators: super specialists pay a price**. Conservation Biology, 18(2), 344-349.

Flohé, L., Brigelius-Flohé, R., Saliou, C., Traber, M. G., Packer, L., 1997. **Redox regulation of NF-kappa B activation**. Free Radical Biology and Medicine, 22(6), 1115-1126.

Flux, J. E., 1994. **World distribution** In **The European rabbits, the history and biology of a successful colonizer** (HV Thompson y CM King, eds.), Oxford University Press, Oxford, 8-21.

Fordham, D. A., Sinclair, R. G., Peacock, D. E., Mutze, G. J., Kovaliski, J., Cassey, P., Capucci, L., Brook, B. W., 2012. **European rabbit survival and recruitment are linked to epidemiological and environmental conditions in their exotic range**. Austral Ecology, 37 (8), 945–957.

Foronda, P., Valladares, B., Lorenzo-Morales, J., Ribas, A., Feliu, C., Casanova, J. C., 2003. **Helminths of the wild rabbit (*Oryctolagus cuniculus*) in Macaronesia**. Journal of Parasitology, 89(5), 952-957.

Foronda, P. R., Figueruelo, E. O., Ortega, A. R., Abreu, N. A., Casanova, J. C., 2005. **Parasites (viruses, coccidia and helminths) of the wild rabbit (*Oryctolagus cuniculus*) introduced to Canary Islands from Iberian Peninsula**. Acta Parasitologica, 50(1), 80-84.

Fouchet, D., Guitton, J. S., Marchandeau, S., Pontier, D., 2008. **Impact of myxomatosis in relation to local persistence in wild rabbit populations: the role of waning immunity and the reproductive period**. Journal of theoretical biology, 250(4), 593-605.

Foyer, C. H., Noctor, G., 2005. **Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context**. Plant, Cell and Environment, 28(8), 1056-1071.

Franzmann, A. W., Schwartz, C. C., 1988. **Evaluating condition of Alaskan black bears with blood profiles**. The Journal of Wildlife Management, 52(1), 63-70.

Frölich, K., Klima, F., Dedek, J., 1998. **Antibodies against rabbit hemorrhagic disease virus in free-ranging red foxes from Germany**. Journal of wildlife diseases, 34(3), 436-442.

Gálvez-Bravo, L. I., 2008. **El conejo europeo (*Oryctolagus cuniculus*) como especie ingeniera de ecosistemas**. PhD Universidad de Alcalá de Henares. Departamento de ecología.

Gálvez-Bravo, L., López-Pintor, A., De Miguel, J. M., Alonso, G., Rueda, M., Rebollo, S., Gómez-Sal, A., 2008. **Ecosystem engineering effects of European rabbits in a Mediterranean habitat** In **Lagomorph Biology**, 125-139.

Gálvez-Bravo, L., Belliure, J., Rebollo, S., 2009. **European rabbits as ecosystem engineers: warrens increase lizard density and diversity**. Biodiversity and Conservation, 18(4), 869-885.

García-Bocanegra, I., Astorga, R. J., Napp, S., Casal, J., Huerta, B., Borge, C., Arenas, A., 2010. **Myxomatosis in wild rabbit: design of control programs in Mediterranean ecosystem**s. Preventive veterinary medicine, 93(1), 42-50.

García-Bocanegra, I., Astorga, R. J., Napp, S., Huerta, B., Carbonero, A., Perea, A., Arenas, A., 2011. **Factors affecting the seroprevalence of lagovirus infection in wild rabbits (*Oryctolagus cuniculus*) in Southern Spain**. The Veterinary Journal, 189(1), 89-94.

García-Fuentes, A., Muñoz-Rodríguez, J. J., Cano-Carmona, E., 2006. **Alteraciones florísticas en los pastizales sometidos a altas densidades de conejos**. Revista del Colegio Oficial de Biólogos de Andalucía **5**, 2–5.

Garner, W., Steinberger, Y., 1989. **A proposed mechanism for the formation of fertile islands in the desert ecosystem**. Journal of arid Environments, 16(3), 257-262.

Gassó, D., Vicente, J., Mentaberre, G., Soriguer, R., Rodríguez, R. J., Navarro-González, N., Tvarijonaviciute, A., Lavín, S., Fernández-Llario, P., Segalés, J., Serrano, E., 2016. **Oxidative Stress in Wild Boars Naturally and Experimentally Infected with *Mycobacterium bovis***. PloS one, 11(9), e0163971.

Gea-Izquierdo, G., Muñoz-Igualada, J., San Miguel-Ayanz, A., 2006. **Rabbit warren distribution in relation to pasture communities in Mediterranean habitats: consequences for management of rabbit populations**. Wildlife Research, 32(8), 723-731.

Gechev, T. S., Van Breusegem, F., Stone, J. M., Denev, I., Laloi, C., 2006. **Reactive oxygen species as signals that modulate plant stress responses and programmed cell death**. BioEssays, 28, 1091-1101.

Genovart, M., Pradel, R., Oro, D., 2012. **Exploiting uncertain ecological fieldwork data with multi-event capture–recapture modelling: an example with bird sex assignment**. Journal of animal ecology, 81(5), 970-977.

Geraldes, A., Ferrand, N., Nachman, M.W., 2006. **Contrasting patterns of introgression at X-linked loci across the hybrid zone between subspecies of the European rabbit (*Oryctolagus cuniculus*)**. Genetics, 173(2), 919-933.

Gershwin, M. E., Beach, R. S., Hurley, L. S., 1985. **Nutrition and immunity**. Academic, Orlando, USA, FL.

Gibb, J. A., 1990. **The European rabbit, *Oryctolagus cuniculus***. Pages 116–120 in J. A.Chapman and J. E. C.Flux, editors. **Rabbit, hares and pikas: status survey and conservation action plan**. IUCN/SCC Lagomorph Specialist Group, Information Press, Oxford , United Kingdom.

Gibbs, H. L., Chiucchi, J. E., 2012. **Inbreeding, body condition, and heterozygosity-fitness correlations in isolated populations of the endangered eastern massasauga rattlesnake (*Sistrurus c. catenatus*)**. Conservation Genetics, 13(4), 1133-1143.

Gillham, M. E., 1955. **Ecology of the Pembrokeshire Islands: III. The effect of grazing on the vegetation.**The Journal of Ecology, 172-206.

Gómez Sal, A., Rey Benayas, J. M., López-Pintor, A., Rebollo, S., 1999. **Role of disturbance in maintaining a savanna-like pattern in Mediterranean *Retama sphaerocarpa* shrubland**. Journal of Vegetation Science, 10(3), 365-370.

Gonçalves, H., Alves, P. C., Rocha, A., 2002. **Seasonal variation in the reproductive activity of the wild rabbit (*Oryctolagus cuniculus algirus*) in a Mediterranean ecosystem**. Wildlife Research, 29(2), 165-173.

Graham, A. L., Cattadori, I. M., Lloyd-Smith, J. O., Ferrari, M. J., Bjørnstad, O. N., 2007. **Transmission consequences of coinfection: cytokines writ large?**. Trends in parasitology, 23(6), 284-291.

Grosbois, V., Tavecchia, G., 2003. **Modeling dispersal with capture–recapture data: disentangling decisions of leaving and settlement**. Ecology, 84(5), 1225-1236.

Guitton, J. S., Devillard, S., Guénézan, M., Fouchet, D., Pontier, D., Marchandeau, S., 2008. **Vaccination of free-living juvenile wild rabbits (*Oryctolagus cuniculus*) against myxomatosis improved their survival**. Preventive veterinary medicine, 84(1), 1-10.

Hall, L. S., Myers, K., 1978. **Variations in the microclimate in rabbit warrens in semi-arid New South Wales**. Austral Ecology, 3(2), 187-194.

Halliwell, B., Gutteridge, J. M. C., 1999. **The chemistry of free radicals and related 'reactive species'**. Free Radical Biology and Medicine, 3, 1-7.

Halliwell, B. H., Gutteridge, J. M. C., 2007. **Free radicals in biology and medicine**, 4th edition. Oxford University Press, Oxford.

Harder, J. D., Kirkpatrick, R. L., 1994. **Physiological methods in wildlife research. Research and management techniques for wildlife and habitats**. 5th edition. The Wildlife Society, Bethesda, Maryland, USA, 275-306.

Harman, E., 1956. **Protein oxidation in aging and age-related diseases**. Gerontology, 11, 298-300.

Heard, H. K., O'Connor, K., Strayer, D. S., 1990. **Molecular analysis of immunosuppression induced by virus replication in lymphocytes**. The Journal of Immunology, 144(10), 3992-3999.

Hellgren, E. C., Vaughan, M. R., Kirkpatrick, R. L., 1989. **Seasonal patterns in physiology and nutrition of black bears in Great Dismal Swamp, Virginia–North Carolina**. Canadian Journal of Zoology, 67(8), 1837-1850.

Hellgren, E. C., Rogers, L. L., Seal, U. S., 1993. **Serum chemistry and hematology of black bears: physiological indices of habitat quality or seasonal patterns?**.Journal of Mammalogy, 74(2), 304-315.

Henning, J., Meers, J., Davies, P. R., Morris, R. S., 2005. **Survival of rabbit haemorrhagic disease virus (RHDV) in the environment**. Epidemiology and Infection, 133(4), 719–730.

Henzell, R. P., Cooke, B. D., Mutze, G. J., 2008. **The future biological control of pest populations of European rabbits, *Oryctolagus cuniculus***. Wildlife Research, 35(7), 633-650.

Hirakawa, H., 2001**. Coprophagy in leporids and other mammalian herbivores**. Mammal Review, 31(1), 61-80.

Hobbs, R. P., Twigg, L. E., 1998. **Coccidia (*Eimeria spp*) of wild rabbits in southwestern Australia**. Australian veterinary journal, 76(3), 209-210.

Hobbs, R. P., Twigg, L. E., Elliot, A. D., Wheeler, A. G., 1999a. **Evaluation of the association of parasitism with mortality of wild European rabbits *Oryctolagus cuniculus* (L.) in southwestern Australia**.The Journal of parasitology, 803-808.

Hobbs, R. P., Twigg, L. E., Elliot, A. D., Wheeler, A. G., 1999b. **Factors influencing the fecal egg and oocyst counts of parasites of wild European rabbits *Oryctolagus cuniculus*** (L.) in Southern Western Australia. The Journal of parasitology, 796-802.

Hockett, B. S., Bicho, N. F., 2000. **The rabbits of Picareiro Cave: small mammal hunting during the Late Upper Palaeolithic in the Portuguese Estremadura.** Journal of Archaeological Science, 27(8), 715-723.

Hockett, B., Haws, J. A., 2002. **Taphonomic and methodological perspectives of leporid hunting during the Upper Paleolithic of the western Mediterranean Basin**. Journal of Archaeological method and Theory, 9(3), 269-302.

Hudson, P. J., Dobson, A. P., Lafferty, K. D., 2006. **Is a healthy ecosystem one that is rich in parasites?** Trends in Ecology and Evolution, 21(7), 381-385.

Hurlbert, S. H., 1984. **Pseudoreplication and the design of ecological field experiments**. Ecological Monographs, 54(2), 187-211.

Hulbert, A. J., Pamplona, R., Buffenstein, R., Buttemer, W.A., 2007. **Life and death: metabolic rate, membrane composition, and life span of animals**. Physiological Reviews, 87(4), 1175–1213.

Iborra, O., Lumaret, J. P., 1997. **Validity limits of the pellet group counts in wild rabbit (*Oryctolagus cuniculus*)**. Mammalia, 61(2), 205-218.

Jeklova, E., Leva, L., Matiasovic, J., Kovarcik, K., Kudlackova, H., Nevorankova, Z., Psikal, I., Faldyna, M., 2008. **Characterisation of immunosuppression in rabbits after infection with myxoma virus**. Veterinary microbiology, 129(1), 117-130.

Johnson, J. B., Omland, K. S., 2004. **Model selection in ecology and evolution**. Trends in ecology and evolution, 19(2), 101-108.

Joubert, L., Lefteriotis, E., Mouchet, J., 1972. **La Myxomatose I y II**. L'Expansion Scientifique, Paris, France.

Kerr, P. J., 1997. **An ELISA for epidemiological studies of myxomatosis: persistence of antibodies to myxoma virus in European rabbits (*Oryctolagus cuniculus*)**.Wildlife Research, 24(1), 53-65.

Kerr, P. J., 2012. **Myxomatosis in Australia and Europe: a model for emerging infectious diseases**. Antiviral Research, 93(3), 387-415.

Kerr, P. J., Donnelly, T. M., 2013. **Viral infections of rabbits**. Veterinary Clinics of North America: Exotic Animal Practice, 16(2), 437-468.

Kerr, P. J., Hone, J., Perrin, L., French, N., Williams, C. K., 2010. **Molecular and serological analysis of the epidemiology of myxoma virus in rabbits**. Veterinary microbiology, 143(2), 167-178.

Kessel, J. F., Prouty, C. C., Meyer, J. W., 1931. **Occurence of infectious myxomatosis in southern California**. Proceedings of the Society for Experimental Biology and Medicine. 28(4), 413–414.

Klebanoff, S. J., Clark, R. A., 1978. **The neutrophil: function and clinical disorders**. North-Holland.

Lachish, S., Knowles, S. C., Alves, R., Wood, M. J., Sheldon, B. C., 2011a. **Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates**. Journal of Animal Ecology, 80(6), 1207-1216.

Lachish, S., Knowles, S. C., Alves, R., Wood, M. J., Sheldon, B. C. 2011b. **Fitness effects of endemic malaria infections in a wild bird population: the importance of ecological structure**. Journal of Animal Ecology, 80(6), 1196-1206.

Lastra, R. G., 2009.**Mecanismos moleculares implicados en la fisiopatología de la enfermedad hemorrágica del conejo, un modelo animal de fallo hepático fulminante**. PhD, Universidad de León.

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guerin, J. L., Marchandeau, S., 2011. **Detection of a new variant of rabbit haemorrhagic disease virus in France. Veterinary Record**, 168, 137–138.

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J. L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. **Emergence of a new lagovirus related to rabbit haemorrhagic disease virus**. Veterinary Research, 44(1), 81.

Le Pendue, J., Abrantes, J., Bertagnoli, S., Guitton, J. S., Le Gall-Reculé, G., Lopes, A. M., Marchandeau, S., Alda, F., Almeida, T., Alves, P. C., Barcena, J., Burmakina, G., Blanco, E., Calvete, C., Cavadini, P., Cooke, B., Dalton, K., Delibes-Mateos, M., Deptula, W., Eden, J. S., Wang, F., Ferreira, C., Ferreira, P., Foronda, P., Gonçalves, D., Gavier-Widén, D., Hall, R., Hukowska-Szematowicz, B., Kerr, P., Kovaliski, J., Lavazza, A., Mahar, J., Malogolovkin, A., Marques, R. M., Marques, S., Martin-Alonso, A., Monterroso, P., Moreno, S., Mutze, G., Neimanis, A., Niedzwiedzka-Rystwej, P., Peacock, D., Parra, F., Rocchi, M., Rouco, C., Ruvoën-Clouet, N., Silva, E., Silvério, D., Strive, T., Thompson, G., Tokarz-Deptula, B., Esteves, P., 2017. **Proposal for a unified classification system and nomenclature of lagoviruses**. Journal of General Virology, 98(7), 1658-1666.

Lebreton, J. D., Burnham, K. P., Clobert, J., Anderson, D. R., 1992. **Modeling survival and testing biological hypotheses using marked animals: a unified approach with case studies**. Ecological monographs, 62(1), 67-118.

Lees, A. C., Bell, D. J., 2008. **A conservation paradox for the 21st century: the European wild rabbit *Oryctolagus cuniculus*, an invasive alien and an endangered native species**. Mammal Review, 38(4), 304-320.

Legendre, P., 2008. **lmodel2: Model II Regression**. R package version 1.6-3.

Lello, J., Boag, B., Hudson, P. J., 2005. **The effect of single and concomitant pathogen infections on condition and fecundity of the wild rabbit (*Oryctolagus cuniculus*)**. International journal for parasitology, 35(14), 1509-1515.

Liu, S. J., Xue, H. P., Pu, B. Q., Qian, N. H., 1984. **A new viral disease in rabbits**. Animal Husbandry and Veterinary Medicine (Xumu yu Shouyi), 16(6), 253-255.

Lloyd, H. G., 1970. **Post-myxomatosis rabbit populations in England and Wales**. European Plant Protection Organisation Publication Series A, 58, 197-215.

Lochmiller, R. L., Deerenberg, C., 2000. **Trade-offs in evolutionary immunology: just what is the cost of immunity?** Oikos, 88(1), 87-98.

Lockley, R. M., 1961. **Social Structure and Stress in the Rabbit Warren. Journal of Animal Ecology** 30(2), 385-423.

Lombardi, L., Fernández, N., Moreno, S., Villafuerte, R., 2003. **Habitat-related differences in rabbit (*Oryctolagus cuniculus*) abundance, distribution and activity**. Journal of Mammalogy, 84(1), 26-36.

López-Martínez, N., 1989. **Revisión sistemática y bioestratigráfica de los Lagomorpha (Mammalia) del Terciario y Cuaternario inferior de España**. PhD Universidad de Madrid. Memoria Museo Paleontológico, Universidad de Zaragoza.

Lykkesfeldt, J., Svendsen, O., 2007. **Oxidants and antioxidants in disease: oxidative stress in farm animals**. The Veterinary Journal, 173(3), 502-511.

Maceda-Veiga, A., Figuerola, J., Martínez-Silvestre, A., Viscor, G., Ferrari, N., Pacheco, M., 2015. **Inside the Redbox: applications of haematology in wildlife monitoring and ecosystem health assessment**. Science of the Total Environment 514, 322–332.

Malo, J. E., Suárez, F., 1995. **Herbivorous mammals as seed dispersers in a Mediterranean dehesa**. Oecologia, 104(2), 246-255.

Malo, J. E., Jiménez, B., Suárez, F., 1995. **Seed bank build-up in small disturbances in a Mediterranean pasture: the contribution of endozoochorous dispersal by rabbits. Ecography**, 18(1), 73-82.

Malo, J. E., Jiménez, B., Suárez, F., 2000. **Herbivory dunging and endozoochorous seed deposition in a Mediterranean dehesa**. Journal of Rangeland Management 53(3), 322–328.

Marchandeau, S., Guenezan, M., Chantal, J., 1995. **Utilisation de la croissance pondérale pour la détermination de l'âge de jeunes lapins de garenne (*Oryctolagus cuniculus*)**. Gibier faune sauvage, 12(4), 289-302.

Marchandeau, S., Boucraut-Baralon, C., 1999. **Épidémiologie de la myxomatose et des caliciviroses apparentées à la VHD dans une population sauvage de lapins de garenne (*Oryctolagus cuniculus*)**. Gibier faune sauvage, 16(1), 65-80.

Marchandeau, S., Aubineau, J., Baudron, P., Blanchet, L., Chauvet, C., Legros, E., Merlet, C., Suret, H., 1999. **La pathologie de Lapin du garenne dans l'Ouest de la France**. Bulletin Mensuel de l'Office National de la Chasse, 245, 10-16.

Marchandeau, S., Pontier, D., Guitton, J. S., Letty, J., Fouchet, D., Aubineau, J., Bertagnoli, S., 2014. **Early infections by myxoma virus of young rabbits (*Oryctolagus cuniculus*) protected by maternal antibodies activate their immune system and enhance herd immunity in wild populations**. Veterinary Research, 45(1), 26.

Marri, V., Richner, H., 2015. **Immune response, oxidative stress and dietary antioxidants in great tit nestlings**. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 179, 192-196.

McColl, K. A., Merchant, J. C., Hardy, J., Cooke, B. D., Robinson, A., Westbury, H. A., 2002. **Evidence for insect transmission of rabbit haemorrhagic disease virus**. Epidemiology and Infection, 129(3), 655-663.

McCord, J. M., Fridovich, I., 1969. **Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein)**. Journal of Biological Chemistry, 244(22), 6049-55.

Meyers, G., Wirblich, C., Thiel, H. J., 1991. **Genomic and subgenomic RNAs of rabbit hemorrhagic disease virus are both protein-linked and packaged into particles**. Virology, 184(2), 677-686.

Milner, J. M., Stien, A., Irvine, R. J., Albon, S. D., Langvatn, R., Ropstad, E., 2003. **Body condition in Svalbard reindeer and the use of blood parameters as indicators of condition and fitness**. Canadian Journal of Zoology, 81(9), 1566-1578.

Mitro, S., Krauss, H., 1993. **Rabbit hemorrhagic disease: a review with special reference to its epizootiology**. European journal of epidemiology, 9(1), 70-78.

Moleon, M., Sanchez-Zapata, J. A., Real, J., García-Charton, J. A., Gil-Sánchez, J. M., Palma, L., Bautista, J., Bayle, J., 2009. **Large-scale spatio-temporal shifts in the diet of a predator mediated by an emerging infectious disease of its main prey**. Journal of Biogeography, 36 (8),1502–1515.

Molina, X., Casanova, J. C., Feliu, C., 1999. **Influence of host weight, sex and reproductive status on helminth parasites of the wild rabbit, *Oryctolagus cuniculus*, in Navarra, Spain**. Journal of helminthology, 73(3), 221-225.

Moller, H., Clapperton, B. K., Fletcher, D. J., 1997. **Density of rabbits (*Oryctolagus cuniculus* L.) in the Mackenzie Basin, South Island, New Zealand**. New Zealand Journal of Ecology, 161-167.

Møller, A. P., Christe, P., Erritzøe, J., Mavarez, J., 1998. **Condition, disease and immune defence**. Oikos, 301-306.

Monaghan, P., Metcalfe, N. B., Torres, R., 2009. **Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation**. Ecology Letters, 12(1), 75-92.

Monnerot, M., Vigne, J. D., Biju-Duval, C., Casane, D., Callou, C., Hardy, C., Mougel, F., Soriguer, R., Dennebouy, N., Mounolou, J. C., 1994. **Rabbit and man: genetic and historic approach**. Genetics Selection Evolution, 26(1), S167.

Moreno, S., Villafuerte, R., 1995. **Traditional management of scrubland for the conservation of rabbits *Oryctolagus cuniculus* and their predators in Doñana National Park, Spain**. Biological Conservation, 73(1), 81-85.

Moreno, S., Villafuerte, R., Cabezas, S., Lombardi, L., 2004. **Wild rabbit restocking for predator conservation in Spain**. Biological Conservation, 118(2), 183-193.

Moreno, S., Beltrán, J. F., Cotilla, I., Kuffner, B., Laffite, R., Jordán, G., Ayala, J., Quintero, C., Jiménez, A., Castro, F., Cabezas, S., Villafuerte, R., 2007. **Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain**. Wildlife Research, 34(8), 652-658.

Morner, T., Obendorf, D. L., Artois, M., Woodford, M. H., 2002. **Surveillance and monitoring of wildlife diseases**. Revue scientifique et technique OIE, 21(1), 67-76.

Moss, B., 1996. **Poxviridae: the viruses and their replication**. In: Knipe, D.M., Howley, P.M. (Eds.), Fields' Virology, 5th ed. Lippincott Williams and Wilkins, Philadelphia, 2905–2945.

Moussa, A., Chasey, D., Lavazza, A., Capucci, L., Šmid, B., Meyers, G., Rossi, C., Thiel, H. J., Vlásak, R., Rønsholt, L., Nowotny, N., McCullough, K., Gavier-Widen, D., 1992. **Haemorrhagic disease of lagomorphs: evidence for a calicivirus**. Veterinary microbiology, 33(1-4), 375-381.

Muñoz, G., 1960. **Anverso Y Reverso de la Mixomatosis**. Servicio Nacional de Pesca Fluvial y Caza Ed., Madrid.

Muñoz Reinoso, J. C., 1993. **Consumo de gálbulos de sabina (*Juniperus phoenicea ssp turbinata guss*, 1891) y dispersión de semillas por el conejo (*Oryctolagus cuniculus* L.) en el parque nacional de Doñana**. Doñana Acta Vertebrata, 20(1), 49-58.

Mutze, G., Bird, P., Cooke, B., Henzell, R., 2008. **Geographic and seasonal variation in the impact of rabbit haemorrhagic disease on European rabbits, *Oryctolagus cuniculus*, and rabbit damage in Australia**. In: Lagomorph Biology. Springer Berlin Heidelberg, 279-293.

Mutze, G., Kovaliski, J., Butler, K., Capucci, L., McPhee, S., 2010. **The effect of rabbit population control programmes on the impact of rabbit haemorrhagic disease in south-eastern Australia**. Journal of Applied Ecology, 47(5), 1137-1146.

Myers, K., Bults, H. G., 1977. **Observations on changes in the quality of food eaten by the wild rabbit**. Australian Journal of Ecology, 2(2), 215-229.

Myers, K., Parer, I., Wood, D., Cooke, B. D., 1994. **The rabbit in Australia**. In **The European Rabbit. The history and biology of a successful colonizer**. Eds: Thompson, K. and King, C.M. Oxford University Press, Oxford, 108-157.

Mykytowycz, R., 1958. **Contact transmission of infectious myxomatosis of the rabbit, *Oryctolagus cuniculus* (L.)**. CSIRO Wildlife Research, 3(1), 1-6.

Mykytowycz, R., 1959. **Effect of infection with myxomatosis virus on the endoparasites of rabbits**. Nature, 183(4660), 555-556.

Mykytowycz, R., 1962. **Epidemiology of coccidiosis (*Eimeria spp*.) in an experimental population of the Australian wild rabbit, *Oryctolagus cuniculus* (L.)**. Parasitology, 52(3-4), 375-395.

Nelson, R. J., Demas, G. E., 1996. **Seasonal changes in immune function**. The Quarterly Review of Biology, 71(4), 511-548.

Norte, A. C., Ramos, J. A., Sousa, J. P., Sheldon, B. C., 2009.**Variation of adult great tit Parus major body condition and blood parameters in relation to sex, age, year and season**. Journal of Ornithology, 150(3), 651–660.

Nussey, D. H., Pemberton, J. M., Pilkington, J. G., Blount, J. D., 2009. **Life history correlates of oxidative damage in a free-living mammal population**. Functional Ecology, 23(4), 809-817.

Ohlinger, V. F., Haas, B., Meyers, G., Weiland, F., Thiel, H. J., 1990. **Identification and characterization of the virus causing rabbit hemorrhagic disease**. Journal of Virology 64(7), 3331–3336.

Ohlinger, V. F., Haas, B., Thiel, H. J., 1993. Rabbit haemorrhagic disease (RHD):characterisation of the causative calicivirus. Veterinary Research 24(2), 103–116.

OIE (World Organisation for Animal Health). 2012.**Technical disease cards**. **http://www.oie.int/en/ourscientific-expertise/specific-information-andrecommendations/ technical-disease-cards/**. Accessed September 2012.

OIE (World Organisation for Animal Health): **Technical disease cards**; 2013 [http://www.oie.int/en/animal-health-in-the-world/technical-disease-cards/].

Oppelt, C., Starkloff, A., Rausch, P., Von Holst, D., Rödel, H. G., 2010. **Major histocompatibility complex variation and age-specific endoparasite load in subadult European rabbits**. Molecular ecology, 19(19), 4155-4167.

Osacar-Jimenez, J. J., Lucientes-Curdi, J., Calvete-Margolles, C., 2001. **Abiotic factors influencing the ecology of wild rabbit fleas in north-eastern Spain**. Medical and veterinary entomology, 15(2), 157-166.

Pacios-Palma, I., Santoro, S., Bertó-Moran, A., Moreno, S., Rouco, C., 2016. **Effects of myxoma virus and rabbit hemorrhagic disease virus on the physiological condition of wild European rabbits: Is blood biochemistry a useful monitoring tool?** Research in Veterinary Science, 109, 129-134.

Pakandl, M., 2009. **Coccidia of rabbit: a review**. Folia Parasitologica, 56(3), 153.

Palomares, F., 2001. **Comparison of 3 methods to estimate rabbit abundance in a Mediterranean environment**.Wildlife Society Bulletin, 578-585.

Palomares, F., Delibes, M., 1993. **Key habitats for Egyptian mongooses in Doñana National Park, south-western Spain**. Journal of Applied Ecology, 30(4), 752-758.

Palomares, F., Gaona, P., Ferreras, P., Delibes, M., 1995. **Positive effects on game species of top predators by controlling smaller predator populations: an example with lynx, mongooses, and rabbits**. Conservation Biology, 9(2), 295-305.

Palomares, F., Delibes, M., Revilla, E., Calzada, J., Fedriani, J. M., 2001. **Spatial ecology of Iberian lynx and abundance of European rabbits in southwestern Spain**. Wildlife Monographs, (148), 1-36.

Pap, P. L., Sesarman, A., Vágási, C. I., Buehler, D. M., Pătraş, L., Versteegh, M. A., Banciu, M., 2014. **No evidence for parasitism-linked changes in immune function or oxidative physiology over the annual cycle of an avian species**. Physiological and Biochemical Zoology, 87, 729–739.

Parer, I., Libke, J. A., 1985. **Distribution of rabbit, *Oryctolagus cuniculus*, warrens in relation to soil type**. Australian Wildlife Research, 12(3), 387-405.

Parkes, J. P., Norbury, G. L., Heyward, R. P., Sullivan, G., 2002. **Epidemiology of rabbit haemorrhagic disease (RHD) in the South Island, New Zealand, 1997–2001**.Wildlife Research, 29(6), 543-555.

Parkes, J. P., Glentworth, B., Sullivan, G., 2008. **Changes in immunity to rabbit haemorrhagic disease virus, and in abundance and rates of increase of wild rabbits in Mackenzie Basin, New Zealand**. Wildlife Research, 35(8), 775-779.

Parra, F., Prieto, M., 1990. **Purification and characterization of a calicivirus as the causative agent of a lethal hemorrhagic disease in rabbits**. Journal of virology, 64(8), 4013-4015.

Pedersen, A. B., Fenton, A., 2007. **Emphasizing the ecology in parasite community ecology**. Trends in ecology and evolution, 22(3), 133-139.

Peeters, J. E., Geeroms, R., Froyman, R., Halen, P., 1981. **Coccidiosis in rabbits: a field study**. Research in veterinary science, 30(3), 328-334.

Peeters, J. E., Pohl, P., Charlier, G., Geeroms, R., Glorieux, B., 1984**. Infectious agents associated with diarrhoea in commercial rabbits: a field study**. In **Annales de recherches vétérinaires,** 15(3), 335-340.

Peig, J., Green, A. J., 2009. **New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method**. Oikos, 118(12), 1883-1891.

Petterson, D., 2001.**The effects of the wild rabbit (*Oryctolagus cuniculus*) on soils and vegetation in semi-arid, south-eastern Spain**. PhD, University of Leeds, School of Biology.

Pinilla, R., Tamajón, R., Muñoz, J. M., 1995. **Vegetación actual**. In **Reconocimiento Biofísico de Espacios Naturales Protegidos, Parque Natural Sierra de Hornachuelos**. Edited by Consejería de Medio Ambiente. Seville, Spain: Junta de Andalucía; 207–247.

Piorno, V., 2006. **Gestión cinegética y conservación del conejo de monte**. PhD Universidad de Vigo. Departamento de Ecología y Biología Animal.

Pradel, R., 2009. **The stakes of capture–recapture models with state uncertainty**. In **Modeling demographic processes in marked population**. Edited by Thomson DL, Cooch EG, Conroy MJ. New York: Springer; 781-795.

Pradel, R., Sanz-Aguilar, A., 2012. **Correction: Modeling Trap-Awareness and Related Phenomena in Capture-Recapture Studies**. PloS one, 7(6).

Prieto, J. M., Fernandez, F., Alvarez, V., Espi, A., García-Marín, J. F., Alvarez, M., Martín, J. M., Parra, F., 2000. **Immunohistochemical localisation of rabbit haemorrhagic disease virus VP-60 antigen in early infection of young and adult rabbits**. Research in veterinary science, 68(2), 181-187.

Prior, R. L., Cao, G., 1999. **In vivo total antioxidant capacity: comparison of different analytical methods 1**. Free Radical Biology and Medicine, 27(11), 1173-1181.

Quan, L. J., Zhang, B., Shi, W. W., Li, H. Y., 2008. **Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network**. Journal of Integrative Plant Biology, 50(1), 2-18.

R Core Team, 2016. **R: A Language and Environment for Statistical Computing**. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Raja-aho, S., Kanerva, M., Eeva, T., Lehikoinen, E., Suorsa, P., Gao, K., Vosloo, D., Nikinmaa, M., 2012. **Seasonal variation in the regulation of redox state and some biotransformation enzyme activities in the barn swallow (*Hirundo rustica L.*)**. Physiological and Biochemical Zoology, 85(2), 148–158.

Ratcliffe, F. N., Myers, K., Fennessy, B. V., Calaby, J. H., 1952. **Myxomatosis in Australia: A step towards the biological control of the rabbit**. Nature, 170(4314), 7–19.

Raynaud, J. P., 1970. **Etude de l'efficacité d'une technique de coproscopie quantitative pour le diagnostic de routine et le contrôle des infestations parasitaires des bovins, ovins, équins et porcins**. Annales de Parasitologie Humaine et Comparee, 45(3), 321-342.

Reglero, M., Vicente, J., Rouco, C., Villafuerte, R., Gortazar, C., 2007. ***Trypanosoma spp*. infection in wild rabbits (*Oryctolagus cuniculus*) during a restocking program in Southern Spain**. Veterinary parasitology, 149(3), 178-184.

Revilla, E., Palomares, F., Fernández, N., 2001. **Characteristics, location and selection of diurnal resting dens by Eurasian badgers (*Meles meles*) in a low density area**. Journal of Zoology, 255(3), 291-299.

Rodak, L., Šmíd, B., Valíček, L., Veselý, T., Štěpánek, J., Hampl, J., Jurak, E., 1990. **Enzyme-linked immunosorbent assay of antibodies to rabbit haemorrhagic disease virus and determination of its major structural proteins**. Journal of General Virology, 71(5), 1075-1080.

Rogers, P. M., 1978 . **Predator-prey relationship between rabbit and lynx in southern Spain**. Terre Vie, 32, 83–87.

Rogers, P. M., Myers, K., 1979. **Ecology of the European wild rabbit, *Oryctolagus cuniculus* (L.), in mediterranean habitats**. **I. Distribution in the Landscape of the Coto Doñana, S. Spain**. Journal of Applied Ecology, 16(3), 691-703.

Rogers, P. M., Arthur, A. D., Soriguer, R. C., 1994. **The rabbit in continental Europe.** In **The European Rabbit. The history and biology of a successful colonizer** Eds: Thompson, K. and King, C.M. Oxford University Press, Oxford, 22-63.

Ross, J., 1986. **The influence of myxomatosis in regulating rabbit numbers. Population dynamics and epidemiology of territorial animals**, 62, 433-441.

Ross, J., Tittensor, A. M., Fox, A., Sanders, M. F., 1989. **Myxomatosis in farmland rabbit populations in England and Wales**. Epidemiology and Infection, 103, 333–357.

Rouco, C., Ferreras, P., Castro, F., Villafuerte, R., 2008. **The effect of exclusion of terrestrial predators on short-term survival of translocated European wild rabbits**. Wildlife research, 35(7), 625-632.

Rouco, C., Villafuerte, R., Castro, F., Ferreras, P., 2011. **Effect of artificial warren size on a restocked European wild rabbit population**. Animal Conservation, 14(2), 117-123.

Rouco, C., Starkloff, A., Delibes-Mateos, M., Schubert, M., Rödel, H. G., 2012. **Differentiation of animals from different age classes by means of pellet size: assessment of a field method in European rabbits**. Mammalian Biology-Zeitschrift für Säugetierkunde, 77(6), 451-454.

Rubolini, D., Colombo, G., Ambrosini, R., Caprioli, M., Clerici, M., Colombo, R., Dalle-Donne, I., Milzani, A., Romano, A., Romano, M., Saino, N., 2012. **Sex-related effects of reproduction on biomarkers of oxidative damage in free-living barn swallows (*Hirundo rustica*)**. PloS one, 7(11), e48955.

Saldaña, A., García-Salgado, G., Rebollo, S., 2007. **European rabbit (*Oryctolagus cuniculus* L.) abundance at a regional scale: controlling factors. In 25 Years of Landscape Ecology: Scientific Principles in Practice** Eds: Bunce, R.G.H., Jongman, R.H.G., Hojas L. and Weel S.). Proceedings of the 7th IALE World Congress, Wageningen, The Netherlands, 375-376.

Sanarelli, G., 1898. **Das myxomatogene virus. Beitrag zum Stadium der Krankheitserreger ausserhalb des Sichtbaren**. Zbl. Bakt, 23, 865-873.

Sanchez-Campos, S., Alvarez, M., Culebras, J. M., Gonzalez-Gallego, J., Tunon, M. J., 2004. **Pathogenic molecular mechanisms in an animal model of fulminant hepatic failure: rabbit hemorrhagic viral disease**. Journal of Laboratory and Clinical Medicine, 144(4), 215-222.

Santoro, S., Pacios, I., Moreno, S., Bertó-Morán, A., Rouco, C., 2014. **Multi-event capture–recapture modeling of host–pathogen dynamics among European rabbit populations exposed to myxoma and Rabbit Hemorrhagic Disease Viruses: common and heterogeneous patterns**. Veterinary Research, 45(1), 39.

Schroeder, M. T., 1987. **Blood chemistry, hematology, and condition evaluation of black bears in northcoastal California. Bears: Their Biology and Management**, 333-349.

Selman, C., J. D. Blount, D. H. Nussey, J. R. Speakman., 2012. **Oxidative damage, ageing, and life-history evolution: where now?** Trends in Ecology and Evolution, 27(10), 570–577.

Senar, J. C., Conroy, M. J., 2004. **Multi-state analysis of the impacts of avian pox on a population of Serins (*Serinus serinus*): the importance of estimating recapture rates**. Animal Biodiversity and Conservation, 27(1), 133-146.

Sies, H., 1991. **Oxidative stress: from basic research to clinical application**. The American Journal of Medicine, 91(3), S31-S38.

Silva, S. M., S., Ferreira, C., Paupério, J., Silva, R. M., Alves, P. C., Lemos, A., 2015. **Coccidiosis in European rabbit (*Oryctolagus cuniculus algirus*) populations in the Iberian Peninsula**. Acta Parasitologica, 60(2), 350-355.

Skalski, J. R., Hoffmann, A., Smith, S. G., 1993. **Testing the significance of individual-and cohort-level covariates in animal survival studies**. In **Marked Individuals in the Study of Bird Population**. Edited by Lebreton JD, North PM. Bäsel, Switzerland: Birkhäuser; 9–28.

Sobey, W. R., 1969. **Selection for resistance to myxomatosis in domestic rabbits (*Oryctolagus cuniculus*)**. The Journal of hygiene, 67(4), 743.

Sorci, G., Faivre, B., 2009. **Inflammation and oxidative stress in vertebrate host–parasite systems**. Philosophical Transactions of the Royal Society of London B: Biological Sciences, 364(1513), 71-83.

Soriguer, R. C., 1981. **Biología y dinámica de una población de conejos (*Oryctolagus cuniculus,* L.) en Andalucía Occidental**. Doñana Acta Vertebrata, número especial.

Soriguer, R. C., Myers, K., 1986. **Morphological, physiological and reproductive features of a wild rabbit-population in Mediterranean Spain under different habitat management.** In **Mammal Review**. River St, Hoboken 07030-5774, NJ USA: Wiley-Blackwell, 16(3-4), 197-197.

Stanford, M. M., Werden, S. J., McFadden, G., 2007. **Myxoma virus in the European rabbit: interactions between the virus and its susceptible host**. Veterinary Research, 38(2), 299-318.

Stiner, M. C., Munro, N. D., 2002. **Approaches to prehistoric diet breadth, demography, and prey ranking systems in time and space**. Journal of Archaeological Method and Theory, 9(2), 181-214.

Stirrat, S. C., 2003. **Body condition and blood chemistry of agile wallabies (*Macropus agilis*) in the wet–dry tropics**. Wildlife Research, 30(1), 59-67.

Stodart, E., 1968a. **Coccidiosis in wild rabbits, *Oryctolagus cuniculus* (L.), at four sites in different climatic regions in eastern Australia I. Relationship with the age of the rabbit**. Australian Journal of Zoology, 16(1), 69-85.

Stodart, E., 1968b. **Coccidiosis in wild rabbits, *Oryctolagus cuniculus* (L.) at four sites in different climatic regions in eastern Australia II**. The relationship of oocyst output to climate and to some aspects of the rabbit's physiology. Australian Journal of Zoology, 16(4), 619-628.

Swindle, E. J., Metcalfe, D. D., 2007. **The role of reactive oxygen species and nitric oxide in mast cell-dependent inflammatory processes**. Immunological Reviews, 217(1), 186-205.

Tanchev, S., Gadjeva, V., Stanilova, S., 2003. **Influence of antigen stimulation on the oxidative stress parameters in outbred and inbred rabbits**. Archives of Physiology and Biochemistry, 111(5), 437-442.

Thannickal, V. J., Fanburg, B. L., 2000. **Reactive oxygen species in cell signaling**. American Journal of Physiology-Lung Cellular and Molecular Physiology, 279(6), L1005-L1028.

Thomas, F., Renaud, F., Guégan, J. F., 2005. **Parasitism and ecosystems**. Oxford University Press on Demand.

Thompson, H. V., King, C. M., 1994. **The European rabbit**. **The history and biology of a successful colonizer**. Oxford University Press, Oxford, United Kingdom.

Thompson, H. V., 1994.**The rabbit in Britain**. In **The European Rabbit. The history and biology of a successful colonizer**. Eds: Thompson, K. and King, C.M. Oxford University Press, Oxford, 64-107.

Tompkins, D. M., Begon, M., 1999. **Parasites can regulate wildlife populations.** Parasitology Today, 15(8), 311-313.

Travaini, A., Delibes, M., Ferreras, P., Palomares, F., 1997. **Diversity, abundance or rare species as a target for the conservation of mammalian carnivores: a case study in Southern Spain**. Biodiversity and Conservation, 6(4), 529-535.

Trout, R. C., Langton, S., Smith, G. C., Haines-Young, R. H., 2000. **Factors affecting the abundance of rabbits (*Oryctolagus cuniculus*) in England and Wales**. Journal of Zoology 252(2), 227-238.

Tunon, M. J., Sanchez-Campos, S., Garcia-Ferreras, J., Alvarez, M., Jorquera, F., Gonzalez-Gallego, J., 2003. **Rabbit hemorrhagic viral disease: characterization of a new animal model of fulminant liver failure**. The Journal of Laboratory and Clinical Medicine, 141(4), 272-278.

van de Crommenacker, J., Horrocks, N. P., Versteegh, M. A., Komdeur, J., Tieleman, B. I., Matson, K. D., 2010. **Effects of immune supplementation and immune challenge on oxidative status and physiology in a model bird: implications for ecologists**. Journal of Experimental Biology, 213(20), 3527-3535.

Varga, I., 1982. **Large-scale management systems and parasite populations: coccidia in rabbits**. Veterinary parasitology, 11(1), 69-84.

Viggers, K. L., Lindenmayer, D. B., Spratt, D. M., 1993. **The importance of disease in reintroduction programmes**. Wildlife Research, 20(5), 687-698.

Villafuerte, R., 1994. **Riesgo de predación y estrategias defensivas del conejo (*Oryctolagus cuniculus*) en el Parque Nacional de Doñana**. Tesis Doctoral, Universidad de Córdoba.

Villafuerte, R., 2002. ***Oryctolagus cuniculus,*** In **Atlas de los Mamíferos Terrestres de España**, edited by Palomo, L. J. and Gisbert, J. Dirección General de Conservación de la Naturaleza-SECEM-SECEMU, Madrid, 464–466.

Villafuerte, R., Kufner, M. B., Delibes, M., Moreno, S., 1993. **Environmental factors influencing the seasonal daily activity of the European rabbit (*Oryctolagus cuniculus*) in a Mediterranean area**. Mammalia, 57(3), 341-348.

Villafuerte, R., Calvete, C., Gortázar, C., Moreno, S., 1994. **First epizootic of rabbit hemorrhagic disease in free living populations of *Oryctolagus cuniculus* at Doñana National Park, Spain**. Journal of Wildlife Diseases, 30(2), 176-179.

Villafuerte, R., Calvete, C., Blanco, J. C., Lucientes, J., 1995. **Incidence of viral hemorrhagic disease in wild rabbit populations in Spain**. Mammalia, 59(4), 651-660.

Villafuerte, R., Viñuela, J., Blanco, J. C., 1998. **Extensive predator persecution caused by population crash in a game species: the case of red kites and rabbits in Spain**. Biological conservation, 84(2), 181-188.

Villafuerte, R., Delibes-Mateos, M., 2007**. El conejo. Atlas y Libro Rojo de los Mamíferos Terrestres de España**. Dirección General para la Biodiversidad-SECEM-SECEMU, Madrid, 490-491.

Villafuerte, R., Castro, F., Ramirez, E., Cotilla, I., Parra, F., Delibes-Mateos, M., Recuerda, P., Rouco, C., 2017. **Large-scale assessment of myxomatosis prevalence in European wild rabbits (*Oryctolagus cuniculus*) 60 years after first outbreak in Spain**. Research in Veterinary Science, 114, 281-286.

Virgós, E., Cabezas-Díaz, S., Lozano, J., 2007. **Is the wild rabbit (*Oryctolagus cuniculus*) a threatened species in Spain? Sociological constraints in the conservation of species**. Biodiversity and Conservation, 16(12), 3489-3504.

von Schantz, T., Bensch, S., Grahn, M., Hasselquist, D., Wittzell, H., 2016. **Good genes, oxidative stress and condition–dependent sexual signals**. Proceedings of the Royal Society. Series B, Biological Sciences, 266(1414), 1-12.

Ward, D., 2005. **Reversing rabbit decline: one of the biggest challenges for nature conservation in Spain and Portugal**. Field Reports. UICN/SSC, Lagomorph Specialist Group.

Watt, A. S., 1981. **Further observations on the effects of excluding rabbits from grassland A in East Anglian Breckland: the pattern of change and factors affecting it (1936-73)**. Journal of Ecology, 69(2), 509-536.

Westcott, D. G., Frossard, J. P., Everest, D., Dastjerdi, A., Duff, J. P., Choudhury, B., 2014. **Incursion of RHDV2-like variant in Great Britain**. Veterinary Record, 174(13), 333-333.

Wheeler, S. H., King, D. R., 1985. **The European rabbit in south-western Australia II. reproduction**. Wildlife Research, 12(2), 197-212.

Wheeler, S.H., King, D.R., Robinson, M.H., 1981. **Habitat and warren utilisation by the european rabbit, *Oryctolagus cuniculus* (L.), as determined by radio-tracking**. Australian Wildlife Research, 8(3), 581-588.

Williams, C. K., Davey, C. C., Moore, R. J., Hinds, L. A., Silvers, L. E., Kerr, P. J., French, N., Hood, G. M., Pech, R. P., Krebs, C. J., 2007. **Population responses to sterility imposed on female European rabbits**. Journal of Applied Ecology, 44(2), 291–301.

Willott, S. J., Miller, A. J., Incoll, L. D., Compton, S. G., 2000. **The contribution of rabbits (*Oryctolagus cuniculus* L.) to soil fertility in semi-arid Spain**. Biology and Fertility of Soils, 31(5), 379-384.

Wobeser, G. A., 2007. **Disease in wild animals**. 53-82. Berlin, German.

Wood, D. H., 1988. **Estimating rabbit density by counting dung pellets**. Wildlife Research, 15(6), 665-671.

Woodroffe, R., 1999. **Managing disease threats to wild mammals**. Animal Conservation, 2(3), 185-193.

Xu, W. Y., 1991. **Viral haemorrhagic disease of rabbits in the People's Republic of China: epidemiology and virus characterisation**. Revue scientifique et technique OIE, 10(2), 393-408.

Xu, Z. J., Chen, W. X., 1989. **Viral hemorrhagic disease in rabbits: a review**. Veterinary Research Communications. 13(3), 205–212.

Yun, C. H., Lillehoj, H. S., Lillehoj, E. P., 2000. **Intestinal immune responses to coccidiosis**. Developmental and Comparative Immunology, 24(2), 303-324.

Zipkin, E. F., Jennelle, C. S., Cooch, E. G., 2010. **A primer on the application of Markov chains to the study of wildlife disease dynamics**. Methods in Ecology and Evolution, 1(2), 192-198.

Zuur, A. F., Ieno, E. N., Smith, G. M., 2007. **Analysing ecological data**. Springer, New York, New York, USA, 672 pp.

Zuur, A. F., Ieno, E. N., Walker ,N. J., Saveliev, A. A., Smith, G. M., 2009. **Mixed effects models and extension in ecology with R**. Springer, New York, New York, USA, 574 pp.

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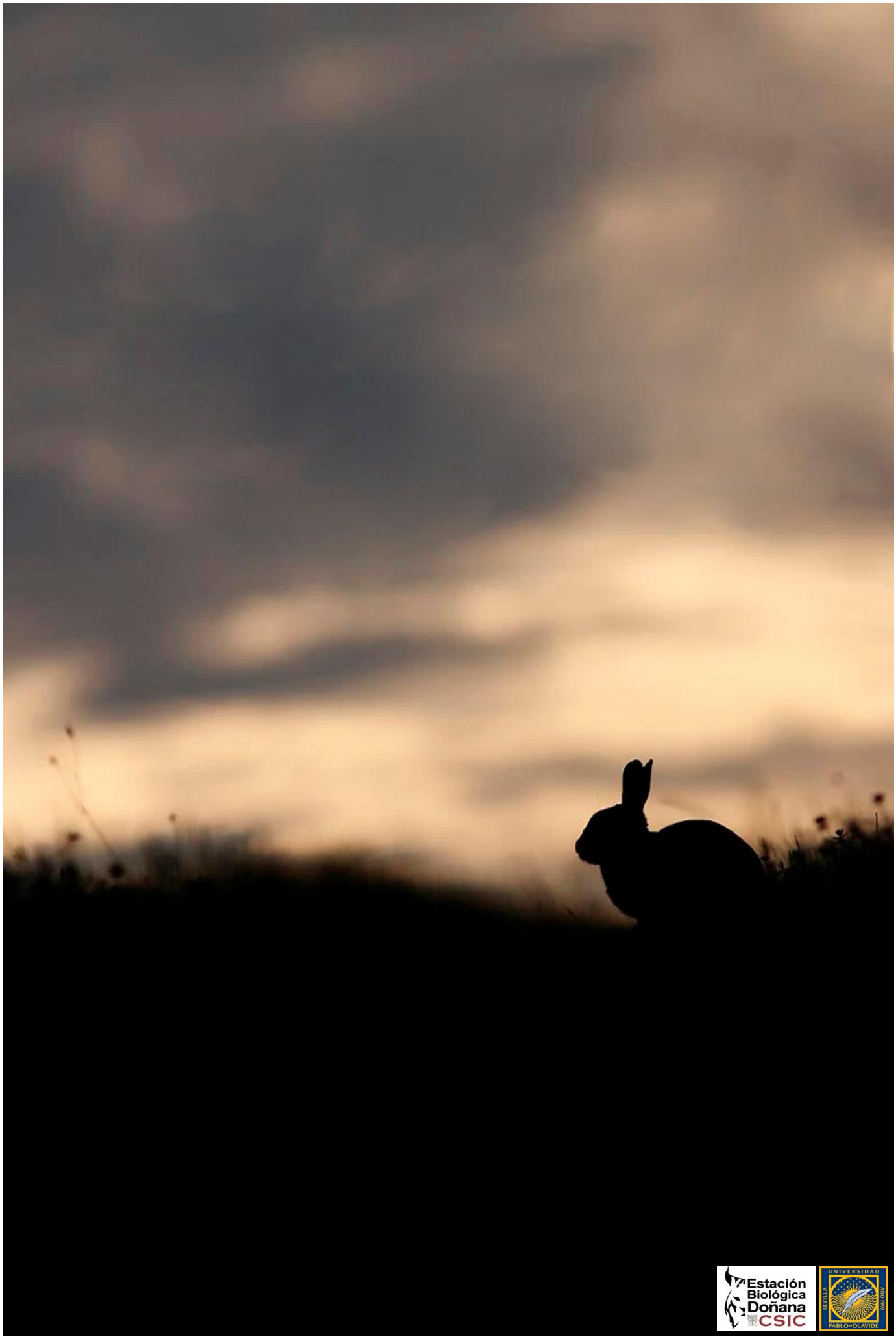
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